The Lipofuscin Fluorophore A2E Mediates Blue Light–Induced Damage to Retinal Pigmented Epithelial Cells

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Purpose. To determine whether the lipofuscin fluorophore A2E participates in blue light–induced damage to retinal pigmented epithelial (RPE) cells.

Methods. Human RPE cells (ARPE-19) accumulated A2E from 10, 50, and 100 μM concentrations in media, the levels of internalized A2E ranging from less than 5 to 64 ng/10^5 cells, as assayed by quantitative high-performance liquid chromatography (HPLC). Restricted zones (0.5-mm diameter spots) of confluent cultures were subsequently exposed to 480 ± 20-nm (blue) or 545 ± 1-nm (green) light for 15 to 60 seconds. Phototoxicity was quantified at various periods after exposure by fluorescence staining of the nuclei of membrane-compromised cells, by TdT-dUTP terminal nick-end labeling (TUNEL) of apoptotic cells and by Annexin V labeling for phosphatidylserine exposure.

Results. Nonviable cells were located in blue light–exposed zones of A2E-containing RPE cells, whereas cells situated outside the illuminated areas remained viable. As shown by fluorescence labeling of the nuclei of membrane-damaged cells and by the presence of TUNEL-positive cells, the numbers of nonviable cells increased with exposure duration and as a function of the concentration of A2E used to load the cells before illumination. The numbers of blue light–induced TUNEL-positive cells also increased in advance of the increase in labeling of membrane-compromised cells, a finding that, together with Annexin V labeling, indicates an apoptotic form of cell death. Conversely, blue light–exposed RPE cells that did not contain A2E remained viable. In addition, illumination with green light resulted in the appearance of substantially fewer nonviable cells.

Conclusions. These studies implicate A2E as an initiator of blue light–induced apoptosis of RPE cells. (*Invest Ophthalmol Vis Sci.* 2000;41:1981–1989)

It is well known that visible and UV radiation can produce photochemical lesions in the neural retina and retinal pigment epithelium (RPE).[1,2] Indeed, RPE cells are particularly susceptible to wavelengths within the blue region of the spectrum.[3–7] In addition to morphologic evidence of cell injury, blue light–induced damage to the RPE in vivo is manifest in blood–retinal barrier dysfunction[8] and in substantial decreases in the apical-to-basal transport of leucine and chloride.[9] Furthermore, studies in vivo have shown that the death of blue light–irradiated RPE cells occurs through apoptotic mechanisms.[10,11] RPE cells in culture are also vulnerable to blue light irradiation, the phototoxicity elicited by 435-nm light being oxygen dependent.[12] Blue light irradiation of RPE cell cultures containing autofluorescent pigment has also been reported to cause lysosomal membrane instability[13] and, although cultures of porcine RPE cells exhibited no morphologic evidence of cellular damage after an 18-hour exposure to blue light, the growth of the RPE cells, in addition to that of endothelial cells and fibroblasts, was reduced significantly.[14]

The chromophore responsible for blue light–induced damage has been debated for some time, with candidates for this role including melanin and mitochondrial respiratory enzymes such as cytochrome-*c* oxidase.[15,16] Indirect evidence also implicates component(s) of RPE lipofuscin as initiators of blue light damage.[15–18] For instance, it has been shown that lipofuscin isolated from RPE can serve as photoinducible generators of singlet oxygen, superoxide anion, and H_2O_2.[19–22] Moreover, in the presence of light and isolated lipofuscin granules, catalase is inactivated, and suspensions of RPE cells and rod outer segments undergo lipid peroxidation.[23] Nevertheless, RPE lipofuscin is a heterogeneous mixture of fluorophores, and the identity of the species responsible for this photoreactivity remains to be determined.

A major hydrophobic component of RPE lipofuscin is the fluorophore A2E, a quaternary pyridinium salt that is the product of the reaction of all-*trans* retinal and ethanolamine.[24–26] Under standard room light or monochromatic blue light (430 nm), A2E is known to interconvert with its double-bond isomer, iso-A2E, with equilibrium reached at an A2E:iso-A2E ratio of 4:1.[27] Because we have detected both A2E and iso-A2E in RPE isolated in the dark from donor human eyes, we assume that this photoisomerization also occurs in vivo.[27] By taking advantage of our ability to synthesize A2E,[27] we have recently demonstrated that when A2E is accumulated by RPE cells in culture, it becomes localized to acidic organelles having a perinuclear distribution and can exert detergent-like activity

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when present in critical concentrations.[28] The uptake of A2E complexed to low density lipoprotein (LDL) has also been shown to result in the alkalinization of lysosomes.[29]

Given that A2E strongly absorbs in the blue region of the spectrum, together with the known susceptibility of RPE to blue light damage, we undertook to investigate A2E as a fluorophore involved in blue light toxicity. To this end, we constructed a cell culture model that allows us to study the effects of blue light on RPE in the presence and absence of intracellular A2E.

METHODS

RPE Cell Cultures

A human adult RPE cell line (ARPE-19; American Type Culture Collection, Manassas, VA) that is devoid of endogenous A2E[28] was maintained as stock cultures in 75-cm^2 flasks with passage weekly. The cells exhibited no evidence of melanin pigmentation. For experimentation, ARPE-19 cells were grown on eight-well plastic chamber slides (Laboratory-Tek; Nunc, Napierville, IL), the bottoms of which were imprinted with an 8 × 8-mm ruled grid, with each square measuring 1 × 1 mm (64 squares). The latter grid was used to record and relocate cell populations according to the variable light conditions to which they had been exposed. The cells were grown as previously described,[28] and all experiments were performed at confluence and within 3 weeks of plating.

A2E Synthesis and Cellular Uptake

A2E was synthesized from all-*trans*-retinal and ethanolamine (2:1 molar ratio), as previously described,[27] and was stored as a stock solution (25 mM) in dimethyl sulfoxide (DMSO).[28] For uptake into confluent cultures of ARPE-19 cells, A2E was delivered in 10-, 50- and 100-μM concentrations in culture media. All experiments included untreated cells. The autofluorescence of cell-associated A2E was detected by epifluorescence microscopy (Axiovert S100; Carl Zeiss, Thornwood, NY) using standard fluorescein isothiocyanate (FITC) filters (460–500-nm excitation, 510–560-nm emission). Internalized A2E was quantified by high-performance liquid chromatography (HPLC), as previously described.[27,28]

Excitation and Emission Spectra of A2E

UV/visible (Vis) radiation spectra of A2E in methanol (6 μM), were measured on a Lambda 40 spectrometer (Perkin–Elmer, Norwalk, CT). Fluorescence emission and excitation spectra of A2E in methanol were measured on a FluoroMax-2 spectrometer (Spex, Edison, NJ). Spectra were obtained using a 1-cm^2 cuvette. Emission spectra were obtained using 400-nm excitation and excitation spectra were obtained while detecting the emission at 600 nm. Maximum absorbance was approximately 0.2.

Illumination

At least 7 days after A2E loading,[28] the cultures were removed from the incubator and immediately exposed to 480 ± 20-nm (blue) or 545 ± 15-nm (green) illumination delivered from a 100-W mercury lamp for 15 to 60 seconds. A single spot of focused illumination (0.5-mm diameter) was applied to the cell population located in an individual square of the locator grid. The irradiance levels of the 480-nm and 545-nm illuminations at full output were measured (Astral AA30; Scientech, Boulder, CO) as 75 mW/mm^2 and 200 mW/mm^2, respectively. Irradiances measured with the addition of an infrared filter were reduced by 16% and 19%, respectively, indicating that these fractions of the radiant energy were delivered as heat. The cultures were exposed to ambient lighting (approximately 75 foot candles) during plating and feeding and while transporting the cells to and from the exposure apparatus. The pH of the media was 7.2 to 7.5. Control conditions included cells not incubated with A2E but exposed to 480-nm light and A2E-containing cells not exposed to 480-nm light.

Fluorescence Assay of Cell Viability

Cell viability was quantified at the indicated times by a two-color fluorescence assay (Molecular Probes, Eugene, OR) in which the nuclei of nonviable cells appear red due to staining by a membrane-impermeant dye (Dead Red nucleic acid stain; Molecular Probes), whereas the nuclei of all cells stained green by a membrane-permeant dye (Syto 10 green fluorescent nucleic acid stain; Molecular Probes). Briefly, the cultures were incubated with the fluorescent dyes (1/500 dilution in HEPES-buffered Hanks' balanced salt solution) for 15 minutes, after which the cells were washed and fixed in 4% glutaraldehyde for 1 hour. For fluorescence detection, the fixed cultures were examined using a fluorescence microscope (Axiovert 100; Zeiss) with the red stain excited at λ 545 ± 15 nm and visualized at 620 ± 30 nm, and the green stain excited at 480 ± 20 nm and visualized at 535 ± 25 nm. The numbers of red-labeled nuclei in the light-exposed fields (0.5 mm diameter) were counted in fluorescence photomicrographs, and background numbers of dead cells, determined by counting red-labeled nuclei in unexposed areas of the cultures, were subtracted. Data are based on counts performed on three to six replicates per experiment. Images were processed for publication using Photoshop 5.0 (Adobe, San Jose, CA).

Detection of Apoptosis-Associated DNA Fragmentation

Cells undergoing apoptosis were detected by labeling for DNA strand breaks by terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick-end labeling (TUNEL).[30] Briefly, cultures were fixed in 2% paraformaldehyde for 30 minutes, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate (2 minutes, 4°C) and incubated in TdT together with dUTP-fluorescein (37°C, 60 minutes; Boehringer–Mannheim, Indianapolis IN). Subsequent incubations were in anti-fluorescein antibody conjugated to alkaline phosphatase (37°C, 30 minutes) followed by fast red (Boehringer Mannheim) as the substrate for alkaline phosphatase detection. The numbers of TUNEL-labeled nuclei in the light-exposed fields (0.5-mm diameter) were counted, and background numbers of labeled cells, determined by counting TUNEL-positive nuclei in unexposed areas of the cultures, were subtracted. Data are based on three to six replicates per experiment. Controls consisted of TUNEL assay of A2E-containing unexposed cells, TUNEL assay of untreated (without A2E), unexposed cells and the incubation of A2E-containing light-exposed cells in labeling solution without TdT.

Annexin V Labeling of Apoptotic Cells

To detect phosphatidylserine (PS) on the external leaflet of plasma membranes, the cultures were incubated for 15 min-

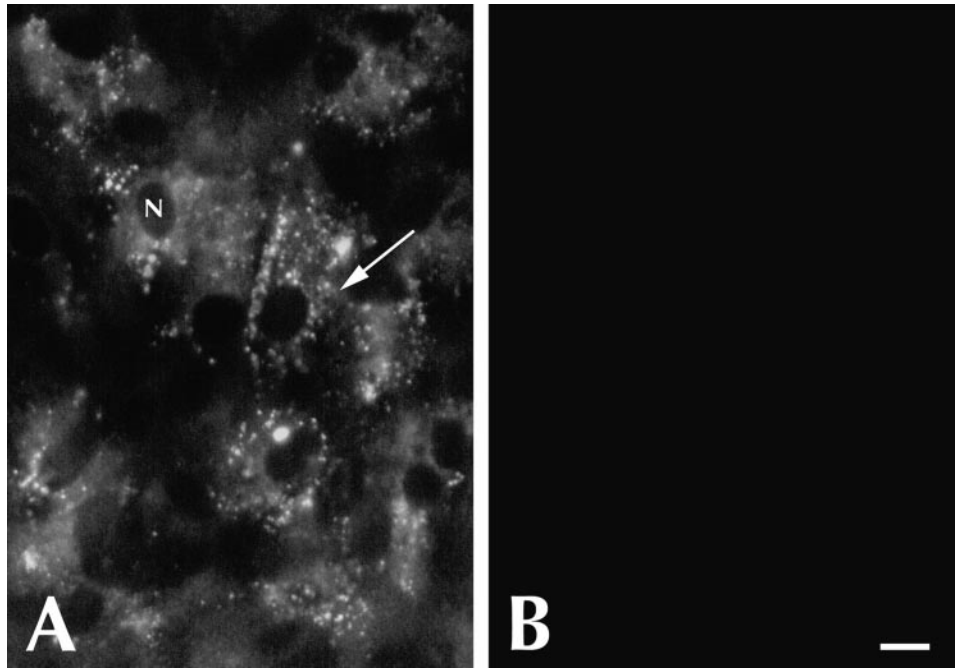

FIGURE 1. Internalization of A2E by RPE cells in culture. (**A**) Epifluorescence detection of A2E accumulation in ARPE-19 cells 7 days after incubation with 100 μM A2E for 2 hours. From inspection of the cells within the plane of focus (*arrow*), it is apparent that the autofluorescence of internalized A2E was associated with intracellular granules. When not in focus, these granules appeared soft-edged and could not be resolved as individual puncta. n, nucleus. (**B**) Control cells not exposed to A2E. Photomicrographs (**A**, **B**) were obtained with FITC-appropriate filters and an exposure duration of 2 seconds. Scale bar, 10 μm.

utes at room temperature in sterile buffer (140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM HEPES/NaOH [pH 7.4]) containing 5 μg/ml FITC-labeled Annexin V (human recombinant; Alexis Biochemicals, San Diego, CA) and Dead Red (1:500 dilution; Molecular Probes). Subsequently, the cultures were washed, and Annexin V–positive/red-negative cells were counted under the fluorescence microscope. Counting of Annexin V–positive cells, which simultaneously excluded the membrane-impermeant red dye, served to control for nonspecific Annexin V binding.

RESULTS

We allowed ARPE-19 cells to accumulate A2E by incubating confluent cultures for 2 hours at 37°C with A2E prepared as 10-, 50-, and 100-μM concentrations in media. Cells in control wells were not loaded with A2E. By epifluorescence microscopy, the internalization of A2E was evidenced by the presence of autofluorescent granules (Fig. 1) that were not present in untreated cells. Moreover, the cells in a single culture varied in the extent to which they accumulated A2E during the 2-hour incubation period. By quantitative HPLC $(UV)^-$, we estimated that the overall levels (mean ± SD) of A2E in the cultured cells were 64 ± 6 ng/10^5 cells at the 100-μM concentration, 23 ± 1 ng/10^5 cells at 50 μM, and less than 5 ng/10^5 cells at 10 μM. These amounts are comparable to A2E levels measured in human RPE isolated from healthy donor eyes (34–134 ng/10^5 cells).[28]

To determine whether A2E-containing RPE cells were susceptible to blue light–induced phototoxicity, 1 week after A2E loading, restricted zones (0.5-mm diameter spots) of confluent monolayers were exposed to 480-nm light. Accordingly, using a fluorescence assay in which the nuclei of nonviable cells fluoresce red, we observed the death of A2E-loaded RPE cells exposed to blue light for varying durations of time (15–60 seconds; Figs. 2 and 3). The labeled nuclei were concentrated in a 0.5-mm diameter zone which corresponded to the area of illumination (Fig. 2A, 2B, 2D, 2E, 2G, 2H). Furthermore, the nonviable cells were uniformly distributed across the diameter of the illuminated field, and A2E-containing cells flanking the exposed area remained viable. Of note, RPE cells that did not contain A2E and that were exposed to blue light of the same intensity for 60 seconds, also remained viable (Fig. 2F). The data presented were obtained in cells maintained either in complete media or in a defined buffer–salt solution (Krebs–Ringer phosphate buffer [KRPG] containing 5.5 mM glucose) during light exposure. Because the phototoxic response to blue light was the same under both conditions, the data were pooled.

Counting of fluorescently labeled nuclei in the illuminated zone (0.5 mm) further revealed that the frequency of membrane-compromised cells increased with time after exposure (Fig. 3). In addition, at 6 and 12 hours after light exposure, the nuclei labeled with red fluorescence were more numerous at the 100-μM concentration of A2E than at the 50-μM concentration, presumably because at the higher loading concentration, a greater number of cells acquired critical concentrations of A2E. Even within a single-culture well, variations in the rate at which cells underwent death may have been due, in part, to differences in the A2E content of individual cells. When cultures were loaded with A2E from a 100-μM concentration in media, exposed to 60 seconds of blue light, and then labeled 18 hours after illumination, the numbers of nonviable red-labeled nuclei reached approximately 75% of the total number of nuclei in the illuminated zone (red-labeled nuclei [mean ± SEM]: 141 ± 18; total nuclei: 189 ± 13, $n = 10$). This value for the proportions of nonviable cells is actually underestimated, however, because the time course of cell death was somewhat variable and in some experiments, cells had begun to detach from the culture dish by 18 hours after exposure. Indeed, the loss of illuminated cells loaded from a 100-μM concentration of A2E accounts for the observation that at 18 hours after illumi-

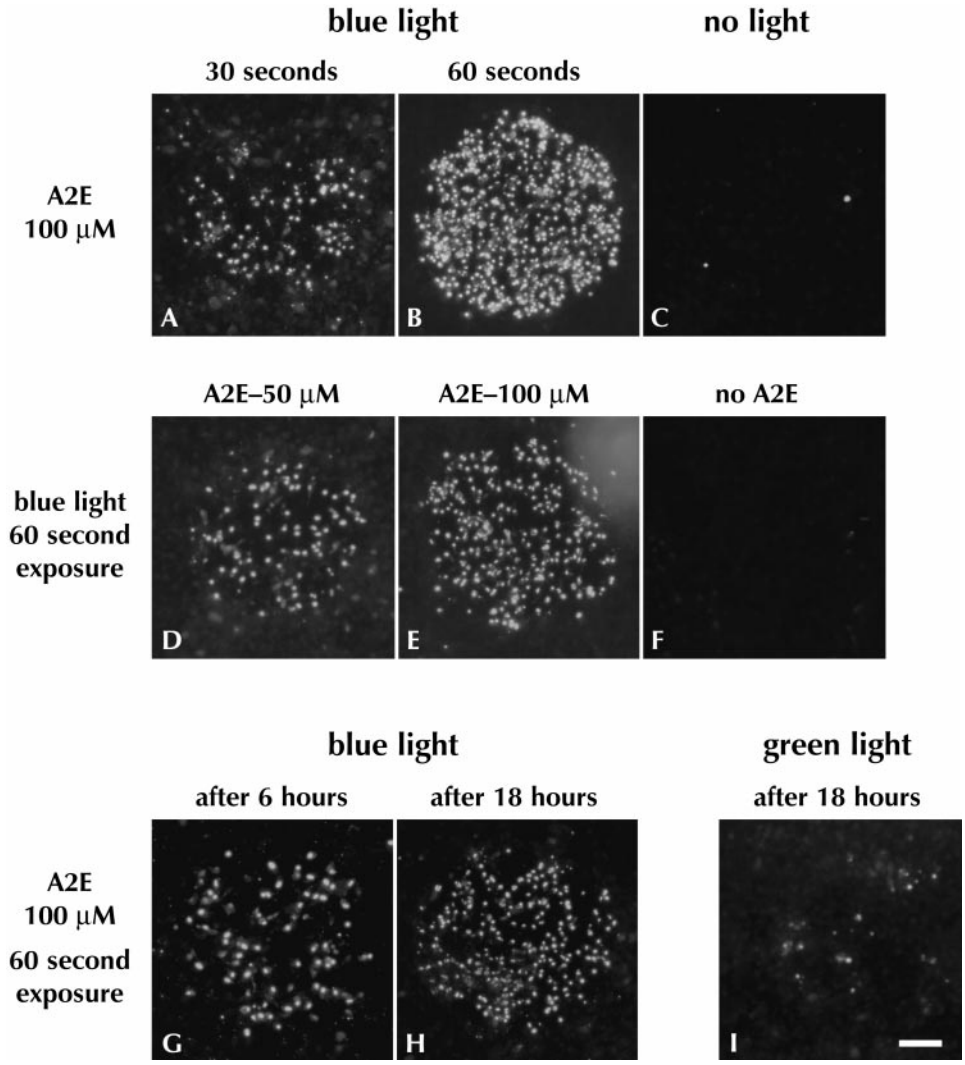

FIGURE 2. ARPE-19 cells containing A2E underwent cell death when exposed to blue light. The numbers of nonviable cells, detected by nuclear labeling with a membrane-impermeant dye, varied with duration of light exposure, concentration of A2E, and time after light exposure. The zones (0.5-mm diameter) of nonviable cells corresponded to the illuminated fields. Viable A2E-loaded cells flanking the zone of illumination excluded red stain. (**A**, **B**, and **C**) A2E-treated (100 μM in media) RPE cells were exposed to 480-nm illumination for 30 or 60 seconds or were not exposed (no light), and nonviable cells were labeled after 18 hours. (**D**, **E**, and **F**) A2E-treated (50 and 100 μM in media) and untreated cultures were exposed to 480-nm illumination for 60 seconds, and nonviable cells were labeled after 18 hours. (**G**, **H**, and **I**) A2E-treated (100 μM) RPE cells were exposed to 480-nm (blue) or 545-nm (green) illumination for 60 seconds and nonviable cells were labeled after 6 or 18 hours, as indicated. Scale bar, 100 μM.

nation, the numbers of red-labeled nuclei at the 50- and 100-μM concentrations of A2E are similar (Fig. 3, 18 hours after light exposure). When the cells had not been loaded with A2E but were exposed to blue light, the incidence of labeled nuclei was not greater than background levels (0–1 nuclei/0.5-mm diameter field) calculated by counting red-labeled nuclei in nonilluminated regions of the cultures.

For cells accumulating A2E from 100-μM concentrations in media, blue light induced death at exposure times of 15 to 60 seconds, with the numbers of nonviable A2E-containing cells within the illuminated region increasing as a function of the exposure time (Fig. 7). Blue light was also significantly more effective in causing lethal damage than green light (Figs. 2, 3), even though with green light the cells were exposed to considerably more radiant energy (blue light, 75 mW/mm^2; green light, 210 mW/mm^2). The differences in cell loss observed with green and blue light can readily be accounted for by both the UV/Vis and excitation spectra of A2E: peak absorbance and excitation occur at approximately 430 nm (blue), with the absorption-emission elicited at 545 nm (green) being approximately 10% of that elicited at 480 nm (blue; Fig. 4).

To ascertain whether cell death occurred by apoptotic mechanisms, we assayed for DNA fragmentation, a process that begins early in apoptosis[31] and that can be detected by the presence of TUNEL-positive nuclei. As shown in Figure 5, blue light–induced TUNEL-positive cells were first detected within the illuminated regions of A2E-loaded cultures 3 hours after light exposure, with the frequency of TUNEL-labeled nuclei increasing at 6 hours after exposure. At 6 hours after blue light exposure, the numbers of TUNEL-labeled nuclei were also greater when the cells had been loaded with A2E from a 100-μM concentration than from a 50-μM concentration (Fig. 6A). Conversely, apoptosis, as detected by TUNEL, was not induced by blue light exposure (60 seconds), when the cells had accumulated A2E from a 10-μM concentration. With blue light exposure, the numbers of TUNEL-positive cells within the illuminated region also increased as a function of the exposure time (15–60 seconds; Figs. 6B and 7). Green light exposure for 60 seconds was, however, associated with the induction of considerably fewer apoptotic nuclei (8.0 $\pm$ 1.4 versus 68.8 $\pm$ 9.7, green and blue light, respectively; Fig. 6C).

Cell death by apoptosis typically proceeds through a programmed set of events, with DNA fragmentation beginning early, whereas the impermeability of the cell membrane is maintained until later stages.[32] Thus, as part of our study we also compared the time course of appearance of TUNEL-positive nuclei with the increase in numbers of red-stained nuclei (Fig. 7). Accordingly, when A2E-containing RPE cells were

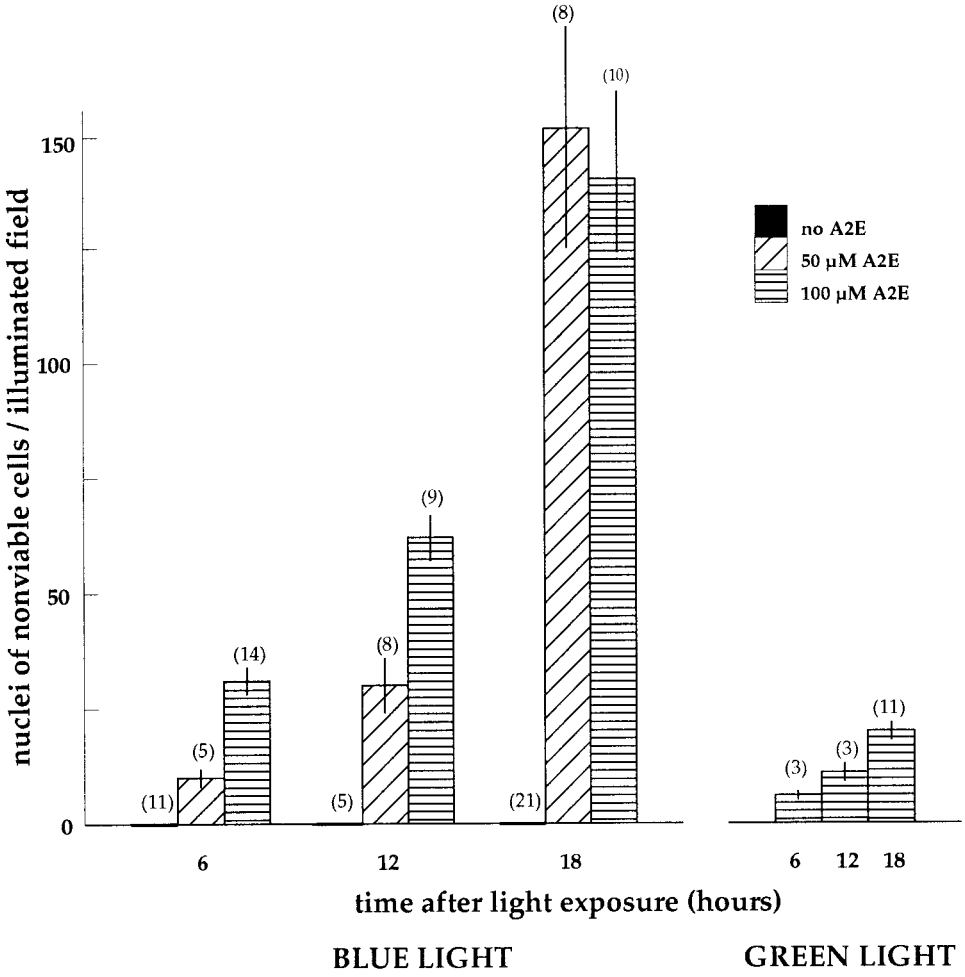

FIGURE 3. Quantitation of nonviable cells after the illumination of A2E-containing ARPE-19 cells in culture. ARPE-19 cells accumulated A2E from 50- and 100-μM concentrations in media. Control cells (without A2E) did not accumulate A2E. Fields of cells (0.5-mm diameter) were exposed to 480-nm (blue) or 545-nm (green) light for 60 seconds. After 6, 12, and 18 hours the nuclei of nonviable cells were fluorescently labeled. Values are means ± SEM. The numbers in parentheses indicate the total number of replicates pooled from one to four experiments. The values for untreated cultures are less than 0.5. Note that the value for 18 hours at 100 μM is underestimated, because in some experiments cells had begun to detach from the culture dish.

exposed to blue light of 15 to 60 seconds' duration, neither TUNEL-positive nor red-stained nuclei were present 1 hour after light exposure. Subsequently, between 3 and 6 hours after light exposure, the numbers of TUNEL-positive and red-labeled nuclei increased, but at both intervals the numbers of TUNEL-positive cells were greater than the numbers of red-stained nuclei. This finding indicates that in the early stages of apoptosis, many cells that were undergoing DNA fragmentation had not yet exhibited altered membrane permeability. This pattern of events is consistent with an apoptotic form of cell death.

The redistribution of PS from the inner to the outer leaflet of the plasma membrane is also an early event in apoptotic cell death[33,34] that occurs after the onset of DNA fragmentation and during the stage when membrane integrity is intact. Externally exposed PS was detected by Annexin V labeling. In addition, to establish that Annexin V was specifically binding to externalized PS rather than to PS within the inner leaflet of the plasma membrane, we simultaneously examined for membrane impermeability, indicated by the exclusion of the membrane-impermeant dye Dead Red. Accordingly, only nuclei that were both Annexin V positive and red negative were counted. Thus, in ARPE-19 cells loaded with A2E from a 100-μM concentration and illuminated with blue light for 60 seconds, Annexin V labeling was readily detectable as a bright membrane fluorescence at 3 hours after exposure. The mean number of Annexin V–positive and red-negative cells in blue light–illuminated fields of A2E-loaded cultures was 20.8 ± 1.9

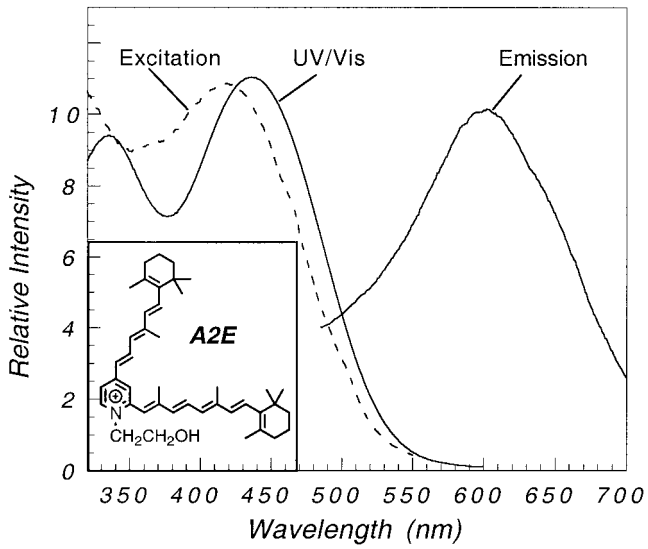

FIGURE 4. UV/Vis, excitation, and emission spectra of A2E in methanol. The absorbance spectrum had a major peak at 435 nm and a lesser peak at 335 nm. The excitation spectrum, monitored at 600-nm emission, was similar in shape with a maximum at 418 nm. A 400-nm excitation wavelength generated a yellow emission centered around 602 nm. *Inset*, structure of A2E.

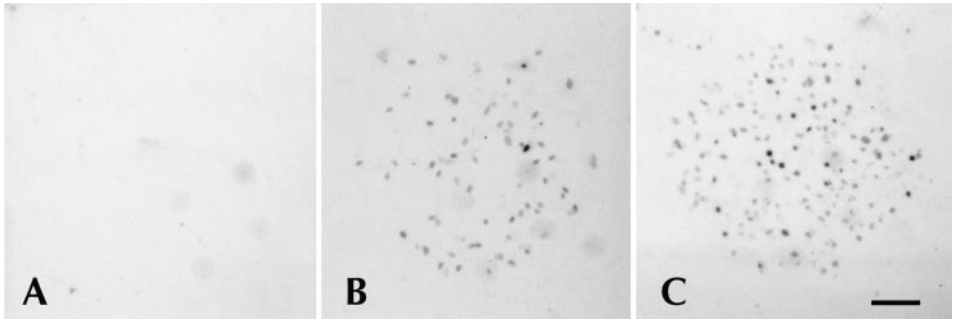

FIGURE 5. Detection of DNA strand breaks in the nuclei of A2E-containing RPE cells exposed to blue light. ARPE-19 cells were untreated (**A**) or were incubated with 100 μM A2E for 2 hours (**B**, **C**). The fields (0.5-mm diameter) of cells were exposed to 480-nm (blue) light for 60 seconds, and after 3 hours (**B**) and 6 hours (**A, C**), nuclei exhibiting DNA fragmentation were labeled by the TUNEL method. Scale bar, 100 μM.

(mean ± SEM; $n = 24$, three experiments). In contrast, no Annexin V–positive and red-negative cells were observed in untreated cultures exposed to blue light for 60 seconds (0 ± 0, $n = 14$, three experiments).

A2E-containing cells that were exposed to blue light also demonstrated nuclear condensation, a morphologic change that is ongoing through apoptosis. The progression of nuclear condensation was apparent when the sizes of the nuclei were compared at 6 hours and 18 hours after blue light irradiation (Figs. 8A, 8B). Moreover, at 18 hours after light exposure apoptotic bodies had also formed (Fig. 8C).

DISCUSSION

These studies implicate A2E as a mediator of blue light damage in the RPE. Thus, using the criteria of the presence of TUNEL-positive cells, exposure of PS on the cell surface, late-onset membrane permeability, nuclear condensation, and apoptotic body formation, we deduce that healthy RPE cells that have amassed A2E at critical concentrations and are exposed to blue light can undergo an apoptotic form of cell death. Central to the conclusion that the photochemical damage is initiated by A2E is the finding that in the absence of internalized A2E, the RPE cells showed no loss of viability in response to blue light exposure. Furthermore, the extent of damage, measured as the numbers of nonviable cells at 6 and 12 hours after light exposure, was directly related to the A2E content of the cells. These results are consistent with previous observations of blue light–induced damage to RPE cells, in vivo.[3–14] Additionally, although the present studies are pertinent to the question of whether blue light–induced injury can originate in the RPE cell rather than be triggered after primary damage to the photoreceptor cells,[16,35] they do not preclude the possibility of direct blue light damage to the photoreceptor cells.

We have previously demonstrated by light and fluorescence confocal microscopy that the A2E that is internalized by cultured cells has a particulate appearance, indicating that it may be localized to spherical organelles.[28] These intracellular granules of A2E also assume a perinuclear distribution, which is typical of lysosomes.[36] Moreover, the colocalization of A2E with a fluorescent acidotropic probe revealed that A2E preferentially accumulates in acidic organelles and may indicate that the compartmentalization of A2E replicates that occurring in vivo. Given that lipofuscin is located within lysosomal storage bodies in the cell, it has been suggested that any adverse effects of its accumulation would be expressed, at least initially,

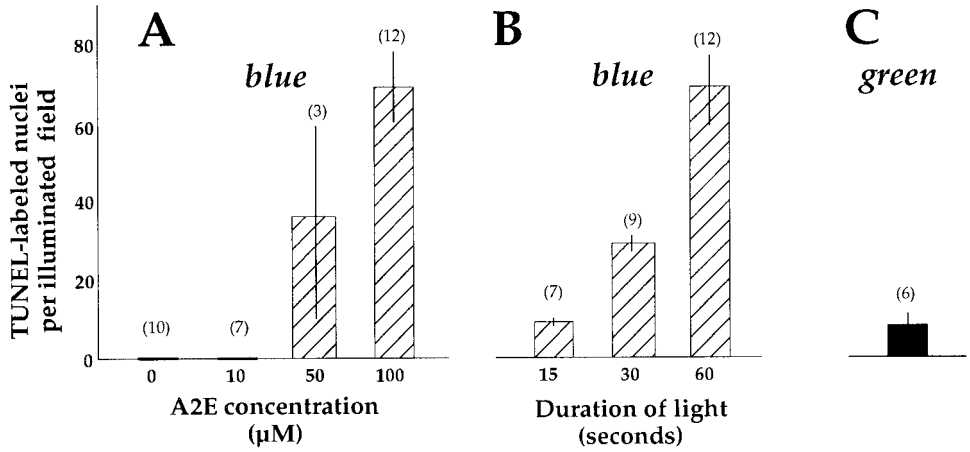

FIGURE 6. Quantitation of TUNEL-positive nuclei 6 hours after exposure of A2E-containing RPE cells to blue light. (**A**) ARPE-19 cells were devoid of A2E (0) or had accumulated A2E from 10-, 50-, and 100-μM concentrations in media, and fields of cells (0.5-mm diameter) were exposed to 480-nm (blue) light for 60 seconds. (**B**) ARPE-19 cells were loaded with A2E from 100-μM concentrations in media, and fields of cells were illuminated with 480-nm (blue) light for 15, 30, or 60 seconds. (**C**) ARPE-19 cells were loaded with A2E from 100-μM concentration in media, and fields of cells were exposed to 545-nm (green) light for 60 seconds. Values are means ± SEM of the numbers of replicates presented in parentheses. The replicates were pooled from one to four experiments.

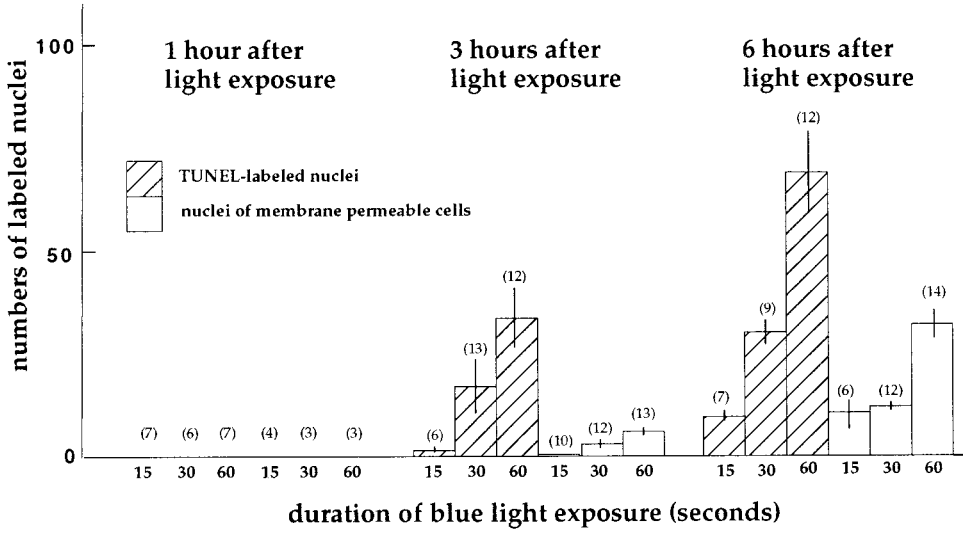

FIGURE 7. The incidence of DNA strand breaks in the nuclei of blue light–damaged A2E-containing RPE cells increased before changes in membrane permeability. ARPE-19 cells were loaded with A2E from 100-μM concentration in media, and the cells were exposed to 480-nm (blue) light for 15, 30, or 60 seconds. One, 3, and 6 hours after exposure, DNA strand breaks were assayed by the TUNEL method, and the nuclei of membrane-permeable cells were fluorescently labeled. Values are means ± SEM of the numbers of labeled nuclei in an illuminated field. The numbers in parentheses indicate the numbers of determinations pooled from one to four experiments. All values at 1 hour after exposure were less than 0.5. The data for TUNEL-positive nuclei 6 hours after light exposure are also presented in Figure 6B.

within these organelles.[23,24] Indeed, in light of the observation that lysosomal pH is elevated in cultured cells that have taken up A2E complexed to LDL,[29] we have previously suggested[28] that A2E may mediate a detergent-like disruption of the membrane-bound adenosine triphosphatase (ATPase) that otherwise maintains lysosomal acidity by pumping protons into the organelle.[37–39] Oxidative damage to the lysosomal membrane may have also been a factor in the phototoxicity visited on A2E-containing cells in the present experiments.

It is said to be a feature of photochemically induced processes, that light intensity and exposure duration are interchangeable with respect to irradiant dose.[5,8,35] There is, however, a departure from this relationship in exposures of long duration and low irradiance, because repair processes in the cell can balance damage mechanisms.[35] In the present study the light intensity was held constant while exposure time was varied, and high irradiance levels with short exposure times were used. Accordingly, the changes observed in our tissue culture model reflected a response that was graded with respect to blue light exposure time. Further, the blue region of the spectrum had a marked ability to induce the death of A2E-containing cells, whereas the longer wavelength green light was considerably less effective. This wavelength dependence of the phototoxicity is consistent with the absorption and excitation spectra of A2E. Green light, however, appears to be more effective at inducing damage to the photoreceptor cell, with the action spectrum of the light damage coinciding with the absorption spectrum of rhodopsin.[40–43]

RPE cell death in animal models of light-induced damage[16] and in cell culture models of chemical oxidant–induced injury[44] has been shown to occur by means of apoptotic mechanisms. Although the photochemical events generating

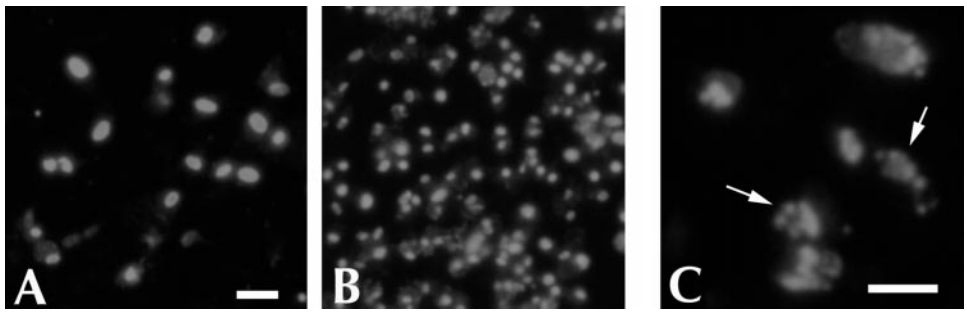

FIGURE 8. Morphologic evidence of apoptosis in blue light–exposed A2E-containing RPE cells. Nuclear condensation progressed between 6 (**A**) and 18 (**B**) hours after blue light exposure (60 second). (**C**) The fragmentation of some nuclei into multiple apoptotic bodies was apparent 18 hours after light exposure. ARPE-19 cells were loaded with A2E from 100-μM concentration in media. Nuclei of membrane-permeable cells were labeled with fluorescent red stain. Scale bar, (**A**, **B**) 25 μM; (**C**) 25 μM.

apoptosis under conditions of blue light injury are not fully understood, reactive oxygen intermediates are likely to serve as mediators. Evidence for the latter assertion is derived from a number of studies demonstrating that the addition of compounds with antioxidant capabilities blocks the onset of light damage.[44–46] In addition, promoters of apoptosis, other than light, are known to elicit oxidative stress.[47] Cellular DNA is considered to be a possible target of reactive oxygen species.[47] For instance, when the death of photoreceptor cells is induced by high intensity light, oxidative damage to DNA, as witnessed by the presence of random single-strand DNA fragmentation, has been shown to precede the onset of apoptosis.[48,49] In this context, it is also notable that lipofuscin has been shown to be a photogenerator of singlet oxygen, superoxide, and hydrogen peroxide, with blue light providing the most efficient radiation.[20–22] It remains to be determined, however, whether oxidative damage is a feature of the blue light–induced RPE damage observed in the present experimental model.

Multiple factors are considered to contribute to the onset of age-related macular degeneration (AMD). Although controversial,[50,51] it has been reported that a subset of patients with nonexudative AMD exhibit heterozygous mutations in the gene encoding for ABCR, the rod-photoreceptor-specific ATP-binding cassette transporter.[52,53] The substrate transported by ABCR is hypothesized to be a phosphatidylethanolamine(PE)-all-*trans*-retinal Schiff-base adduct.[54] It is suggested that the function of ABCR is to flip this Schiff-base adduct from the luminal to the cytosolic face of the outer segment disc membrane, thereby facilitating the reduction of all-*trans*-retinal by all-*trans*-retinol dehydrogenase.[54,55] Significantly, we have previously proposed that this same PE-all-*trans*-retinal Schiff-base adduct is generated as the first step in the biogenesis of A2E,[27] the second step being Schiff base formation with a second molecule of retinal to form a phosphatidyl-pyridinium bisretinoid (A2-PE) that would eventually be hydrolyzed to A2E.[27,56,57] In light of this, it is reasonable to suppose that the consequences of reduced ABCR transport may be an accumulation of the PE-all-*trans*-retinal Schiff-base adduct[54,55] and A2-PE, within the outer segment. This situation would ultimately promote the deposition of A2E in RPE cells because of the role of the RPE cell in phagocytosing packets of outer segment membrane. Mutations in both alleles of ABCR are also known to cause Stargardt disease,[58,59] a macular degeneration of juvenile onset that is characterized by a pronounced accumulation of lipofuscin[60] and progressive atrophy of the RPE.[61] It is notable that mice with a null mutation in ABCR have recently been shown to exhibit accentuated levels of A2E in the RPE.[55] Nevertheless, A2E has not been quantitated in patients with Stargardt disease, nor in individuals with an ABCR-associated autosomal recessive form of retinitis pigmentosa.[62,63]

The amassing of A2E and possibly other components of lipofuscin by the RPE cell is not a benign consequence of aging and disease. In addition to the observations made in the present study, we have previously shown that when A2E accumulates to critical concentrations in cultured ARPE-19 cells, it can behave as an amphiphilic detergent and perturb membrane integrity.[28] The finding in the present work that blue light damage exhibited a dependence on A2E concentration is compatible with the concept that RPE lipofuscin must reach critical levels, above which disease is manifest. Nevertheless, although the accumulation of lipofuscin by aging RPE cells is greatest in the macula,[64,65] evidence for a causal link between lipofuscin and AMD has not been found. Similarly, whether chronic or acute light damage contributes to the pathogenesis of AMD remains to be determined, because epidemiologic studies concerned with the relationship between light exposure (UV and visible radiation) and AMD have been inconclusive.[66,67]

References

1. Noell WK. Possible mechanisms of photoreceptor damage by light in mammalian eyes. *Vis Res.* 1980;20:1163–1171.
2. Marshall J, Greenstein V, Kline D, Owsley C, Werner JS. Optical radiation and the aged eye. In: Waxler M, Hitchins VM, eds. *Optical Radiation and Visual Health*. Boca Raton, FL: CRC Press; 1986: 103–124.
3. Ham WT, Ruffolo JJ, Mueller HA, Clarke AM, Moon ME. Histologic analysis of photochemical lesions produced in rhesus retina by short-wavelength light. *Invest Ophthalmol Vis Sci.* 1978;17:1029–1035.
4. Ham WT, Mueller HA, Ruffolo JJ, et al. Basic mechanisms underlying production of photochemical lesions in the mammalian retina. *Curr Eye Res.* 1984;3:165–174.
5. Ham WT, Allen RG, Feeney–Burns L, et al. The involvement of the retinal pigment epithelium. In: Waxler M, Hitchins VM, eds. *Optical Radiation and Visual Health*. Boca Raton, FL: CRC Press; 1986:43–67.
6. Tso MOM, Fine BS. Repair and late degeneration of the primate foveola after injury by argon laser. *Invest Ophthalmol Vis Sci.* 1979;18:447–461.
7. Busch EM, Gorgels TGMF, van Norren D. Temporal sequence of changes in rat retina after UV-A and blue light exposure. *Vision Res.* 1999;39:1233–1247.
8. Putting BJ, van Best JA, Vrensen GFJM, Oosterhuis. Blue light-induced dysfunction of the blood-retinal barrier at the pigment epithelium in albino versus pigmented rabbits. *Exp Eye Res.* 1994; 58:31–40.
9. Pautler EL, Morita M, Beezley D. Reversible and irreversible blue light damage to the isolated mammalian pigment epithelium. In: LaVail MM, Anderson RE, Hollyfield JG, eds. *Inherited and Environmental Induced Retinal Degenerations*. New York: Alan R. Liss; 1989:555–567.
10. Hafezi F, Marti A, Munz K, Reme CE. Light-induced apoptosis: Differential timing in the retina and pigment epithelium. *Exp Eye Res.* 1997;64:963–970.
11. Reme CE, Weller M, Szczesny P, et al. Light induced apoptosis in the rat retina in vivo. Morphological features, threshold and time course. In: Anderson RE, LaVail MM, Hollyfield JG, *Degenerative Diseases of the Retina*. New York: Plenum Press; 1995:19–24.
12. Crockett RS, Lawwill T. Oxygen dependence of damage by 435 nm light in cultured retinal epithelium. *Curr Eye Res.* 1984;3:209–215.
13. Wihlmark U, Wrigstad A, Roberg K, Nilsson SE, Brunk UT. Lipofuscin accumulation in cultured retinal pigment epithelial cells causes enhanced sensitivity to blue light irradiation. *Free Radic Biol Med.* 1997;22:1229–1234.
14. Dorey CK, Delori FC, Akeo K. Growth of cultured RPE and endothelial cells is inhibited by blue light but not green or red light. *Curr Eye Res.* 1990;9:549–559.
15. Boulton M. Melanin and the retinal pigment epithelium. In: Marmor MF, Wolfensberger TJ, eds. *The Retinal Pigment Epithelium*. New York: Oxford University Press; 1998:68–85.
16. Remé CE, Hafez F, Marti A, Munz K, Reinboth JJ. Light damage to the retina and retinal pigment epithelium. In: Marmor MF, Wolfensberger TJ, eds. *The Retinal Pigment Epithelium*. New York: Oxford University Press; 1998:563–586.
17. Eldred GE. Lipofuscin and other lysosomal storage deposits in the retinal pigment epithelium. In: Marmor MF, Wolfensberger TJ, eds. *The Retinal Pigment Epithelium*. New York: Oxford University Press; 1998:651–668.
18. Dillon J. Photophysics and photobiology of the eye. *J Photochem Photobiol B.* 1991;10:23–40.
19. Gaillard ER, Atherton SJ, Eldred G, Dillon J. Photophysical studies on human retinal lipofuscin. *Photochem Photobiol.* 1995;61:448–453.

20. Boulton M, Dontsov A, Jarvis–Evans J, Ostrovski H, Svistunencko D. Lipofuscin is a photoinducible free radical generator. *J Photochem Photobiol B.* 1993;19:201–204.
21. Rozanowska M, Jarvis–Evans J, Korytowski W, Booulton ME, Burke JM, Sarna T. Blue light-induced reactivity of retinal age pigment. *J Biol Chem.* 1995;270:18825–18830.
22. Rozanowska M, Wessels J, Boulton M, et al. Blue light induced singlet oxygen generation by retinal lipofuscin in non-polar media. *Free Rad Biol Med.* 1998;24:1107–1112.
23. Wassell J, Davies S, Bardsley W, Boulton M. The photoreactivity of the retinal age pigment lipofuscin. *J Biol Chem.* 1999;34:23828–23832.
24. Eldred G, Lasky MR. Retinal age pigments generated by self-assembling lysosomotropic detergents. *Nature.* 1993;361:724–726.
25. Sakai N, Decatur J, Nakanishi K. Ocular age pigment "A2E": an unprecedented pyridinium bisretinoid. *J Am Chem Soc.* 1996;118: 1559–1560.
26. Ren RX-F, Sakai N, Nakanishi K. Total synthesis of the ocular age pigment A2E: a convergent pathway. *J Am Chem Soc.* 1997;119: 3619–3620.
27. Parish CA, Hashimoto M, Nakanishi K, Dillon J, Sparrow JR. Isolation and one-step preparation of A2E and iso-A2E, fluorophores from human retinal pigment epithelium. *Proc Natl Acad Sci USA.* 1998;95:14609–14613.
28. Sparrow JR, Parish CA, Nakanishi K. A2E. a lipofuscin fluorophore, in human retinal pigmented epithelial cells in culture. *Invest Ophthalmol Vis Sci.* 1999;40:2988–2995.
29. Holz FG, Schutt F, Kopitz J, et al. Inhibition of lysososomal degradative functions in RPE cells by a retinoid component of lipofuscin. *Invest Ophthalmol Vis Sci.* 1999;40:737–743.
30. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol.* 1992;119:493–501.
31. Schwartzman RA, Cidlowski JA. Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocr Rev.* 1993; 1993:14:133–151.
32. Darzynkiewicz Z, Bruno S, Del Bino G, et al. Features of apoptotic cells measured by flow cytometry. *Cytometry.* 1992;13:795–808.
33. Koopman G, Reutelingsperger CPM, Kuijten GAM, Keehnen RMJ, Pals ST, van Oers MHJ. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood.* 1994;1994:84:1415–1420.
34. Boersma AWM, Nooter K, Oostrum RG, Stoter G. Quantification of apoptotic cells with fluorescein isothiocyanate-labeled Annexin V in Chinese hamster ovary cell cultures treated with cisplatin. *Cytometry.* 1996;24:123–130.
35. Mellerio J. Light effects on the retina. In: Albert DM, Jakobiec FA, eds. *Principles and Practice of Ophthalmology: Basic Sciences*. Philadelphia: WB Saunders; 1994:1326–1345.
36. Heuser J. Changes in lysosome shape and distribution correlated with changes in cytoplasmic pH. *J Cell Biol.* 1989;108:855–864.
37. Hayashi H, Niinobe S, Matsumoto Y, Suga T. Effects of Triton WR-1339 on lipoprotein lipolytic activity and lipid content of rat liver lysosomes. *J Biochem (Tokyo).* 1981;89:573–579.
38. Ohkuma S, Moriyama Y, Takano T. Identification and characterization of a proton pump on lysosomes by fluorescein isothiocyanate-dextran fluorescence. *Proc Natl Acad Sci USA.* 1982;79:2758–2762.
39. Mellman I, Fuchs R, Helenius A. Acidification of the endocytic and exocytic pathways. *Annu Rev Biochem.* 1986;55:663–700.
40. Noell WK, Walker VS, Kang BS, Berman S. Retinal damage by light in rats. *Invest Ophthalmol.* 1966;5:450–473.
41. Shahinfar S, Edward DP, Tso MOM. A pathologic study of photoreceptor cell death in retinal photic injury. *Curr Eye Res.* 1991; 10:47–59.
42. Williams TP, Howell WL. Action spectrum of retinal light damage in albino rats. *Invest Ophthalmol Vis Sci.* 1983;24:285–287.
43. Organisciak DT, Darrow RM, Bicknell IR, Jiang Y-L, Pickford M, Blanks JC. Protection against retinal light damage by natural and synthetic antioxidants. In: Hollyfield JG, Anderson RE, La Vail MM, eds. *Retinal Degenerations*. Boca Raton, FL: CRC Press; 1991:189–199.
44. Cai J, Wu M, Nelson KC, Sternberg P, Jones DP. Oxidant-induced apoptosis in cultured human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 1999;40:959–966.
45. Organisciak DT, Kutty RK, Leffak M, et al. Oxidative damage and responses in retinal nuclei arising from intense light exposure. In: Anderson RE, LaVail MM, Hollyfield JG, eds. *Degenerative Diseases of the Retina*. 1995:9–17.
46. Organisciak DT, Winkler BS. Retinal light damage: practical and theoretical considerations. In: Osborne NN, Chader GJ, eds. *Progress in Retinal and Eye Research.* Oxford, UK: Pergamon Press; 1994:1–29.
47. Buttke TM, Sandstrom PA. Oxidative stress as a mediator of apoptosis. *Immunol Today.* 1994:7–10.
48. Specht S, Leffak M, Darrow RM, Organisciak DT. Damage to rat retinal DNA induced in vivo by visible light. *Photochem Photobiol.* 1999;69:91–98.
49. Organisciak DT, Darrow RA, Barsalou L, Darrow RM, Lininger LA. Light-induced damage in the retina: differential effects of dimethylthiourea on photoreceptor survival, apoptosis and DNA oxidation. *Photchem Photobiol.* 1999;70:261–268.
50. Stone EM, Webster AR, Vandernburgh K, et al. Allelic variation in abcr associated with Stargardt-disease but not age-related macular degeneration. *Nat Genet.* 20:328–329.
51. De La Paz M, Guy VK, Abou–Donia S, et al. Analysis of the Stargardt disease gene (ABCR) in age-related macular degeneration. *Ophthalmology.* 1999;106:1531–1536.
52. Allikmets R, Schroyer NF, Singh N, et al. Mutation of the Stargardt disease gene (ABCR) in age-related macular degeneration. Science 277 1805–1807.
53. Shroyer NF, Lewis RA, Alllikmets R, et al. The rod photoreceptor ATP-binding cassette transporter gene, ABCR, and retinal disease: from monogenic to multifactorial. *Vision Res.* 1999;39:2537–2544.
54. Sun H, Molday RS, Nathans J. Retinal stimulates ATP hydrolysis by purified and reconstituted ABCR, the photoreceptor-specific ATP-binding cassette transporter responsible for Stargardt's disease. *J Biol Chem.* 1999;274:8269–8281.
55. Weng J, Mata NL, Azarian SM, Tzekov RT, Birch DG, Travis GH. Insights into the function of rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in abcr knockout mice. *Cell.* 1999;98:13–23.
56. Eldred GE. Lipofuscin fluorophore inhibits lysosomal protein degradation and may cause early stages of macular degeneration. *Gerontology.* 1995;41:15–28.
57. Katz ML, Gao C-L, Rice LM. Formation of lipofuscin-like fluorophores by reaction of retinal with photoreceptor outer segments and liposomes. *Mech Ageing Dev.* 1996;92:159–174.
58. Allikmets R, Singh N, Su H, et al. A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Stargardt's macular dystrophy. *Nat Genet.* 1997;15:236–246.
59. Lewis RA, Shroyer NF, Singh N, et al. Genotype/phenotype analysis of a photoreceptor-specific ATP-binding cassette transporter gene, ABCR, in Stargardt's disease. *Am J Hum Genet.* 1999;64:422–434.
60. Delori FC, Staurenghi G, Arend O, Dorey CK, Goger DG, Weiter JJ. In vivo measurement of lipofuscin in Stargardt's disease-fundus flavimaculatus. *Invest Ophthalmol Vis Sci.* 1995;36:2327–2331.
61. Lee BL, Heckenlively JR. Stargardt's disease and fundus flavimaculatus. In: Guyer DR, Yannuzzi LA, Chang S, Shields JA, Green GR, eds, *Retina-Vitreous-Macula*. Philadelphia: WB Saunders, 1999:978–988.
62. Martinez–Mir A, Paloma E, Allikmets R, et al. Retinitis pigmentosa caused by a homozygous mutation in the Stargardt disease gene *ABCR*. *Nat Genet.* 1998;18:11–12.
63. Cremers FP, van de Pol DJ, van Driel M, et al., Autosomal recessive retinitis pigmentosa and cone-rod dystrophy caused by splice site mutations in the Stargardt's disease gene ABCR. *Hum Mol Genet.* 1998;7:355–362.
64. Feeney–Burns L, Hilderbrand ES, Eldridge S. Aging human RPE: morphometric analysis of macular, equatorial and peripheral cells. *Invest Ophthalmol Vis Sci.* 1984;25:195–200.
65. Dorey CK, Wu G, Ebenstein D, Garsd A, Weiter JJ. Cell loss in the aging retina. *Invest Ophthalmol Vis Sci.* 1989;30:1691–1699.
66. West SK, Rosenthal FS, Bressler NM. Exposure to sunlight and other risk factors for age-related macular degeneration. *Arch Ophthalmol.* 1989;107:875–879.
67. Taylor H, West S, Munoz B. The long term effects of visible light on the eye. *Arch Ophthalmol.* 1992;110:99–104.