Photodamage to Human RPE Cells by A2-E, a Retinoid Component of Lipofuscin

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PURPOSE. A fluorescent component of lipofuscin, A2-E (N-retinylidene-N-retinylethanolamine) has been shown to impair lysosomal function and to increase the intralysosomal pH of human retinal pigment epithelial (RPE) cells. In addition to its lysosomotropic properties A2-E is known to be photoreactive. The purpose of this study was to determine the phototoxic potential of A2-E on RPE cells.

METHODS. A2-E (synthesized by coupling all-trans-retinaldehyde to ethanolamine) was complexed to low-density lipoprotein (LDL) to allow for specific loading of the lysosomal compartment. Human RPE cell cultures were loaded with the A2-E-LDL complex four times within 2 weeks. A2-E accumulation was confirmed by fluorescence microscopy and flow cytometry analysis. Acridine orange staining allowed assessment of lysosomal integrity and intralysosomal pH. Exposure of A2-E–fed cells to light resulted in a significant loss of cell viability by 72 hours, which was not observed in either RPE cells maintained in the dark or A2-E–free cultures exposed to light. Toxicity was associated with a loss of lysosomal integrity.

RESULTS. Fluorescence microscopy and flow cytometry analysis demonstrated that the intralysosomal accumulation of A2-E in cultured RPE cells increased with the number of feedings. Acridine orange staining confirmed that the A2-E was located in the lysosomal compartment and induced an elevation of intralysosomal pH. Exposure of A2-E–fed cells to light resulted in a significant loss of cell viability by 72 hours, which was not observed in either RPE cells maintained in the dark or A2-E–free cultures exposed to light. Toxicity was associated with a loss of lysosomal integrity.

CONCLUSIONS. A2-E is detrimental to RPE cell function by a variety of mechanisms: inhibition of lysosomal degradative capacity, loss of membrane integrity, and phototoxicity. Such mechanisms could contribute to retinal aging as well as retinal diseases associated with excessive lipofuscin accumulation—for example, age-related macular degeneration and Stargardt’s disease. (Invest Ophthalmol Vis Sci. 2000;41:2303–2308)

lipofuscin accumulates in postmitotic RPE cells with age, and high levels of this pigment are associated with various degenerative and hereditary diseases including age-related macular degeneration,1–9 Best’s10,11 and Stargardt’s disease.12–14 The major substrate for lipofuscin is considered to be the ingested tips of photoreceptor outer segments, which are rich in lipids and retinoid compounds.15,16 Whereas the role of lipofuscin in retinal aging and disease has yet to be confirmed, studies have demonstrated that lipofuscin is a potent photoinducible generator of reactive oxygen species.17,18

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Lipofuscin represents a mixture of various biomolecules including at least 10 different fluorophores.21,22 A major fluorophore of lipofuscin has been identified as the Schiff-base reaction product N-retinylidene-N-retinylethanolamine (A2-E).23–26 A2-E has been shown to impair lysosomal degradative functions of RPE cells in vitro by elevating the intralysosomal pH.27 In addition, a number of studies have reported that lipofuscin is a photoinducible generator of reactive oxygen species.17,18,22,28 With this in mind, we have investigated the photoreactivity of A2-E in a cellular model in which A2-E–loaded RPE cell cultures are exposed to light.

METHODS

Synthesis of A2-E

A2-E was synthesized by coupling all-trans retinaldehyde to ethanolamine (2:1), exactly according to the protocol of Eldred and Lasky.24 The reaction product was extensively purified by sequential thin-layer chromatography, again following established protocols of Eldred and Lasky.24 and Reinboth et al.29 Although the yield of the whole procedure is relatively low, the
final product was found to be of excellent purity. In analytical high-performance thin-layer chromatography on silica plates using an established 11-component primary developing system, a single band was detected under white light, and a single autofluorescent band was observed under 366-nm illumination. High-performance liquid chromatography analysis of the product using the procedure of Parish et al. with photometric detection at 320 nm and 434 nm and fluorescence detection (excitation 420 nm; emission 605 nm) confirmed the absence of contaminations in our A2-E preparation. The UV spectra and fluorescent properties of our synthetic A2-E corresponded to those reported by other investigators. A2-E was complexed with low-density lipoprotein (LDL), by the addition of 7 nanomoles A2-E to 5 mg LDL particles (Sigma, Munich, Germany) in 1 ml culture medium, and incubated at 37°C for 2 hours. Approximately 90% of the A2-E-associated fluorescence was found in the lipoprotein fraction when the A2-E-LDL complex was analyzed by ultracentrifugation.

RPE Cell Culture

Human RPE cells were isolated and grown in Ham’s F10 medium supplemented with 20% fetal calf serum as previously described. RPE cultures between passage 3 and 7 were grown to confluence on either 13-mm glass coverslips for microscopic examination or 24-well plates for flow cytometry analysis and cell viability studies.

A2-E Loading of RPE Cells

RPE cultures were loaded with the A2-E-LDL complex (10 μg/ml Ham’s F10 medium + 2% fetal calf serum) twice weekly for 2 weeks. Controls were run with medium alone or medium containing LDL without A2-E.

Analysis of A2-E Accumulation by Fluorescence Microscopy and Flow Cytometry

Intracellular fluorescence was assessed using a fluorescence microscope (Vanox; Olympus, Tokyo, Japan; excitation 450–490 nm, emission >510 nm) at various times after feeding. The accumulation of autofluorescent A2-E was confirmed as previously described using a fluorophotometric flow cytometer (FACS analyzer; Becton Dickinson, Franklin Lakes, NJ; excitation 488 nm, emission 530 nm), to determine the mean autofluorescence percent population of RPE.

Design of the Lighting Conditions

An acute sunlight source (Sol 500; Honle UV, Birmingham, UK) with emission spectrum from 350 to 1200 nm was used for the light exposures. Spectrum filters (Lee, Westbrook, CT) were placed in front of the light source to provide a broad band emission spectrum of 390 to 550 nm, with an irradiance of 2.8 mW/cm^2. The height of the light source was positioned to ensure that the cells were exposed to a constant temperature of 37°C.

Light Exposure Protocol

Confluent RPE cultures in 24-well plates were treated four times with SF10PF medium alone (a modified Ham’s F10 that had no identified photosensitizers, such as phenol red, tryptophan, tyrosine, riboflavin, and folic acid). Immediately before experimentation, the cells were washed three times with SF10PF medium and the medium replaced with SP10F medium containing 20 mM HEPES buffer at pH 7.4. For each experiment, cultures were divided in two; one set of cultures (cells with and without A2E) were wrapped in aluminum foil (dark maintained), whereas the other set remained open to light (light exposed). Both sets were then placed under the light source and exposed to light at 390 to 550 nm for various times up to 144 hours. Cultures were assessed in the following assays.

Acridine Orange Staining

RPE cells grown on coverslips were used for AO staining. At each time point, the medium was aspirated from the wells, and 1 ml SF10PF medium containing 5 μg/ml AO and 10 μg/ml ethidium bromide was added. The cells were incubated for 5 minutes at 37°C in 5% CO₂ before they were washed twice in SF10PF. The distribution of fluorescence was monitored by fluorescence microscopy.

Cell Viability Assays

Assays were performed at four time points (0, 48, 96, and 144 hours), and the experiment was repeated twice. At each time point the cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and photographs were taken to document the morphologic changes during the light exposures (Axiovert 35; Carl Zeiss, Oberkochen, Germany). The MTT assay was performed as described by Mosmann. In brief, at each time point, medium was aspirated from the wells and 250 μl MTT (1 mg/ml prepared in SF10PF) was gently added to each well. The cells were incubated for 3 hours at 37°C in 5% CO₂, after which the MTT was aspirated and 250 μl acidified isopropanol (0.04 M HCl) added to solubilize the reduced blue formazan crystals. Aliquots were transferred to a 96-well plate and the absorbance measured at a test filter of 590 nm and a reference filter of 630 nm on a 96-well plate reader. Results were analyzed using the following equation:

Viability as a percentage of control = (absorbance of sample cells) / (absorbance of control cells) × 100%

where sample cells are dark-maintained and light-exposed cells, and control cells are viability of cells immediately before the beginning of the experiment.

Statistics

Student’s t-test and analysis of variance were undertaken using the Sigmfit data analysis program (University of Manchester). P < 0.05 was considered significant.

RESULTS

A2-E Accumulation in RPE Cells

A2-E accumulated as discrete autofluorescent intracytoplasmic inclusions (Fig. 1E). The number and fluorescence intensity of these inclusions appeared to increase with increasing dose. By contrast RPE cells receiving either LDL or medium alone dem-
onstrated only weak background fluorescence. Acridine orange staining indicated that most of the A2-E was intralysosomal (Fig. 1A, 1B). RPE cells without A2-E showed red lysosomes, which is consistent with an acidic lysosomal pH (Fig. 1A). A2-E-treated cells showed in the majority of green lysosomes, representing a neutral or alkaline pH (Fig. 1B). These results are in agreement with studies using Lysosensor yellow-blue (Molecular Probes, Eugene, OR).27

Flow cytometry further confirmed the dose-dependent intracellular increase in fluorescence (Fig. 2). Fluorescence increased more than 80-fold after four feedings during a 2-week period. There was no significant increase in overall cellular fluorescence in cultures exposed to LDL or medium alone.

Phototoxicity of A2-E

A2-E–fed cells demonstrated a time-dependent decrease in cell viability, monitored by the MTT assay (Fig. 3). Slight toxicity was observed in both dark-maintained cells fed A2-E and light-exposed cells in the absence of A2-E after 4 days’ incubation, but this was always significantly less than that observed in A2-E cells exposed to light ($P < 0.001$). After 5 days, viability was reduced 70% compared with untreated control cultures and by 40% compared with A2-E–loaded cells maintained in the dark. There was no difference between the effect of light on RPE cells in either the presence or absence of LDL (data not shown).
The cell viability data were supported by microscopic observation that demonstrated cell damage in A2-E cells exposed to light. These cells demonstrated membrane blebbing, rounded refractile bodies, cytoplasmic vacuolization, condensation and detachment from the substrate (Fig. 1D). The extent of damage increased with the duration of the experiment. No cell damage was observed in A2-E–free cells maintained in the dark throughout the time course of the experiment (Fig. 1C).

Staining with acridine orange demonstrated a loss of lysosomal stability in A2-E–fed cultures exposed to light in which the discrete staining observed in non–light-exposed controls was replaced by diffuse green staining throughout the cytoplasm (Figs. 1A, 1B).

**DISCUSSION**

The results in this study demonstrate that A2-E is phototoxic to human RPE cells confirming its potential as a significant contributor to the photoreactivity of lipofuscin. It appears that A2-E has the potential to cause RPE dysfunction through at least two mechanisms: light-independent inhibition of lysosomal function and phototoxicity. We have previously demonstrated that the lipofuscin fluorophor A2-E inhibits lysosomal degradative functions by elevating intralysosomal pH in cultured RPE cells in the absence of light. Intralysosomal degradative enzymatic capacity is inhibited secondary to this pH shift, because activity of lysosomal hydrolases depends on intralysosomal pH.

When we initiated the study, the original protocol of Eldred and Lasky was the only procedure available for A2-E synthesis. Recently, Parish et al. have published a greatly improved procedure for preparation of A2-E, which produces excellent yields and omits laborious purification by the use of a single-column chromatographic step for preparation of the final product.

The ability of lipofuscin to generate reactive oxygen species (e.g., superoxide anion, singlet oxygen, hydroxyl radical, and lipid peroxides) is well documented. It has more recently been demonstrated that lipofuscin is responsible for light-dependent lipid peroxidation, enzyme inactivation and RPE cell death, with the effect being greater at the shorter visible wavelength end of the visible spectrum.

Taking the data from this and other studies, it is reasonable to suggest that A2-E is the component of lipofuscin responsible for these observations. Others have demonstrated that A2-E is photoreactive and has the capacity to generate a variety of reactive oxygen species, which are known to damage mem-
branes, proteins, and nucleic acids. However, it is unclear whether A2-E is the major photoreactive component of lipofuscin; certainly, the published data suggest that it is not.22,36

We have previously confirmed that A2-E conjugated to LDL accumulates within the lysosomal compartment.27 In this study, we have demonstrated that there is a cumulative uptake of A2-E dependent on the number of feedings. Control cells without any treatment show acidic lysosomal organelles as confirmed by acridine orange staining.

Acridine orange, a meta-chromic chromophore forms an equilibrium within the cell between a charged and uncharged form. It traverses the lysosomal membrane in an uncharged form, but once inside it becomes highly charged because of the low pH and is unable to efflux back to the cytosol. At low-pH acridine orange fluoresces red, whereas at neutral pHi, it fluoresces green when viewed by fluorescence microscopy. It is evident that uptake of A2-E results in a shift toward a neutral pHi in the lysosomal compartment that exhibits green fluorescence. However, if these cultures are light exposed, there is disruption of the lysosomes, and green fluorescence is observed throughout the cytoplasm. This loss of lysosomal integrity has similarly been reported for light-exposed RPE cells fed lipofuscin granules20 and suggests that the loss culminates in cell death.

The observation that light alone (in the absence of A2-E) is detrimental to RPE survival has previously been reported in cultured cells.37 However, this effect was minimal compared with the combination of light and A2-E. LDL, the transport vehicle for A2-E into the lysosomes, does not affect the cellular metabolism. Light exposition with and without LDL incubation caused the same degree of reduction in cell viability. The extent of A2-E’s role in retinal aging and disease is currently unclear. However, evidence for a role for A2-E in retinal degeneration is supported by the findings of Weng et al.38 who studied ABCR knockout mice. These animals are without a specific rim protein because of the loss of the encoding gene and show an enhanced accumulation of toxic A2-E within RPE cells. This rim protein is assumed to function as an outwardly directed flipase for A2-E. Its defect would lead to an accumulation of A2-E within the RPE cells. The ABCR mutation was first found in patients with Stargardt’s disease in which the RPE shows an excessive lipofuscin accumulation at a young age.39 Further studies are necessary to determine the pathophysiological role that A2-E plays in retinal diseases associated with excessive lipofuscin accumulation (e.g., Best’s disease,10,11 Stargardt’s disease,12–14 and age-related macular degeneration15).

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References


