ACCUMULATION OF LIPOFUSCIN WITHIN RETINAL PIGMENT EPITHELIAL CELLS RESULTS IN ENHANCED SENSITIVITY TO PHOTO-OXIDATION.

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Age-related macular degeneration, cell culture, lipofuscin, lysosomes, oxygen radicals, photo-oxidation, retinal pigment epithelium.

Abstract

Retinal pigment epithelial (RPE) cells are largely postmitotic. They continuously phagocytose the outer tips of the photoreceptor outer segments (POS). Over the life span of an individual, this activity results, although surprisingly slowly, in the intralysomal accumulation of lipofuscin, or age-pigment. Native lipofuscin shows orange-red autofluorescence when exposed to blue light. The loss of energy resulting from the conversion of excitatory blue light into emitted orange-red light may induce photo-oxidative reactions. We exposed neonatal rabbit RPE cells *in culture* to purified POS from cow eyes. The material were either native or peroxidized by irradiation with UV-light before being added to the RPE cultures. Lipofuscin accumulation was studied by transmission electron microscopy and measured by microfluorometric registration of its autofluorescence. Cells exposed to peroxidized POS accumulated much more lipofuscin than those exposed to native POS, indicating that peroxidized outer segements are not digestable by lysosomal enzymes. Furthermore, lipofuscin-loaded RPE cells were considerable more sensitive to visible blue light than unloaded control cells. The former ones showed lysosomal membrane destabilization with ensuing leakage of lytic enzymes and eventually cell death. We suggest that photo-oxidation of lysosomal membranes surrounding accumulated lipofuscin may be of importance for the development of age-related macular degeneration.

Introduction

Retinal pigment epithelial (RPE) cells are essential supporters of the light-sensitive photoreceptors of the retina. The tips of the photoreceptor outer segments (POS) are shed daily and phagocytosed by the RPE cells [1, 2]. The phagocytosed POS is then degraded within the acidic vacuolar apparatus of the RPE cells, although not completely. There is, thus, a slowly ongoing accumulation of lipofuscin that by old age may become a dominant part of the RPE cell cytoplasm, especially within the macular area [3]. Lipofuscin, or age pigment, is situated within secondary lysosomes and seems to form as a result of extra- and

intralysosomal peroxidative, iron catalyzed, alterations of protein- and lipid-rich material under degradation [4].

A well known physical property of lipofuscin, both formaldehyde-fixed and native, is its yellowish-reddish autofluorescence when irradiated by blue light [5, 6, 7]. Such energy-transformation is known to induce photo-oxidative processes, since present oxygen would be activated to singlet oxygen or superoxide [8]. Consequently, aged and lipofuscin-rich RPE cells of the retina might be subjected to peroxidative damage of the lysosomal membranes surrounding the accumulated lipofuscin when normally exposed to blue light. That, in turn, might lead to leakage to the cytosol of degrading hydrolytic lysosomal enzymes. Hypothetically, severe cellular degeneration, or even cell death, might be the result of light-exposure to lipofuscin-loaded RPE cells.

We have previously shown that RPE cells may rapidly become converted to artificially aged, lipofuscin-rich, ones by being exposed *in culture* to synthetic lipofuscin, obtained by exposing POS for several hours to UV light [9]. RPE cells readily phagocytose the synthetic lipofuscin, if added to the medium, and incorporate it within their acidic vacuolar apparatuses, where it, due to its polymeric structure, cannot be degraded.

Artificially aged, lipofuscin-loaded RPE cells in culture may consequently be an ideal model system for the study of the proposed light-destabilisation of the acidic vacuolar apparatus.

Material and Methods

Preparation of cell cultures

After a lethal intraperitoneal dose (30 mg) of pentobarbital-sodium, the eyes from newborn pigmented rabbits were enucleated and transferred into a Petri dish. Orbital tissues was removed, and the eyes rinsed three times in +4°C HBSS (calcium-and magnesium-free Hanks´ balanced salt solution). The anterior segement of the bulbs and the retinas were removed. The eyecup was incubated in a trypsin solution (0.05% trypsin - 0.2% EDTA in calcium-and magnesium-free modified Puch´s saline A) for one hr at +37°C. RPE cells were separated from the eyecups by irrigation in HBSS, using a Pasteur pipette. The suspensions were stored on ice during the preparation, which was performed using a dissecting microscope, and centrifuged at 100 g for 10 min. Tissue fragments not recognized as RPE cells were eliminated. The cell suspension from each eye was transferred into a separate 35 mm polystyrene dish (Costar) containing MEM (minimal essential medium, Sigma) with L-glutamine, 10% FCS (fetal calf serum, Sigma) and Penicillin-Streptomycin (Sigma). When confluent, the primary cultures, kept at 37°C in 5% CO_2 in humidified air, were divided into three groups; (i) cells fed approximately 2 x 10^7 peroxidized POS (see below) for one week, (ii) cells daily fed the same approximate number of native POS for up to three weeks, and (iii) control cells kept in culture under normal conditions for up to three weeks (Figure 1).

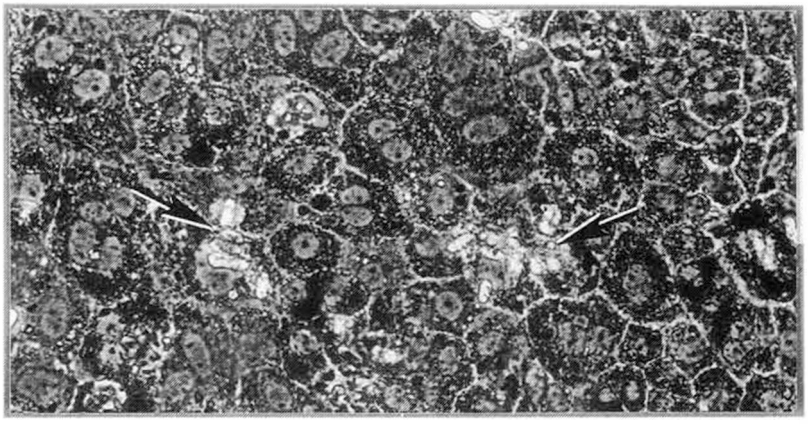

Fig. 1. *A confluent culture of rabbit RPE cells. Arrows show POS added to the culture. Inverted phase contrast microscopy. Bar: 30μm.*

Preparation of native and peroxidized POS

A suspension of photoreceptor outer segments (POS) was prepared in a procedure similar to that of Hall [10]. The retina was removed from adult bovine eyes within 4 hrs after death and transferred to HBSS. The retinal tissue was trypsinized on ice (see above) and fragmented by a magnetic stirrer for 15 min. Large tissue fragments were eliminated by precipitation at +4°C for 10 min and the supernatant was centrifuged at 800 g for another 10 min. The sample was rinsed, and recentrifuged, twice in HBSS. Tubes, each containing approximately 2 x 10^7 native POS, were stored at -70°C.

From a suspension of 1 x 10^8 POS/ml HBSS, 12.5 ml aliquots were transferred to Petri dishes (Ø 90 mm) and exposed to UV-light from above, without lids, in a laminar air-flow cabinet for time-periods up to 18 hrs. The suspension dried up during the irradiation, finally completely. The remains were again made up to 12.5 ml with double-destilled water and suspended by sonification on ice for 20 min. The final, peroxidized POS-material showed a strong yellow autofluorescence when excited at 450 nm. The amount of TBARS, formed during the UV-irradiation-period, was followed [11].

Measurement of lipofuscin-specific autofluorescence

A trypsin-solution (0.25% trypsin in Gibco solution A) was added to the RPE-cultures and the cells were transferred into 35 mm dishes containing 22 mm Ø round glass coverslips. Following 1 hr, allowed for settling, the cells were fixed for 20 min at room-temperature in 4% formaldehyde, made fresh from paraformaldehyde, in PBS. After a rinse in PBS, the coverslips were inverted over PBS-filled excavations of micro-culture slides and the lipofuscin-specific autofluorescence measured from individual cells, using a cytofluorometer based on a Nikon Microphot SA and a Nikon P102 photometer interfaced

to a Macintosh computer, as previously described [7, 12]. A Nikon B-2A filter set containing a 450-490 nm excitation filter, a dichromatic beam-splitter with 50% reflection at 510 nm, and a barrier-filter with its edge at 520 nm on the emission side, was used. All non-lipofuscin-specific autofluorescence was eliminated by an additional 550 nm barrier filter in the beam path. Background fluorescence was automatically substracted. The mean auto-fluorescence/cell was calculated from 100 randomly selected cells of each sample. Data from four or five different samples were used for statistical calculations by the Student´s t-test.

Transmission electron microscopy

Preparation for TEM was performed as described previously [13-15]. Cells were, thus, fixed for 2 hrs in 2% glutaraldehyde at 300 mOsm effective osmotic pressure, post-fixed in OsO_4, stained *en block* by UAc during dehydration, and embedded in Epon. A Jeol 2000-EX electron microscope was used.

Photo-oxidation of RPE-cells

RPE cell-cultures, fed peroxidized POS daily for a week (and their unfed controls) were exposed to blue light for up to 20 min. The growth medium was replaced by PBS during the irradiation and the cultures were exposed from above, without any covering lids, to light from a fluorescence microscope-lamp-house carrying a halogen lamp. The light was filtered through a 2 mm BG 12 blue- and a heat-absorbing filter. Following light-exposures for up to 20 min, the cultures were either; (i) directly subjected to staining with the lysosomotropic weak base acridine-orange, in order to evaluate lysosomal membrane stability (see below), or; (ii) re-established under ordinary culture conditions for another 4 hrs, before being evaluated for cell viability (see below).

Measurements of lysosomal membrane stability and cell viability

Cells, fed peroxidized POS and unexposed controls, were grown on round cover-slips and exposed or not to blue light. They were then vitally stained with the lysosomotropic, fluorescent weak base, acridine-orange as described before [16-18]. Subsequently the coverslips were inverted in PBS on micro-culture slides and red- and green fluorescences were measured, using an MPV III computerized microfluorometeric system [11, 12]. The theoretical background of the procedure has been given before [19]; red fluorescence indicates acridine-orange in high concentrations within the acidic vacuolar apparatus, while green fluorescence reflects cytosolic acridine-orange in less concentrated form.

Cell viability was evaluated using the delayed trypan blue dye exclusion test and was expressed as the percentage of non-stained (living) cells [20-22].

Results

TBA-tests of POS under UV-irradiation showed increasing TBARS during the first 4 hrs followed by decreasing amounts indicating spontaneous decomposition of formed peroxides (Figure 2).

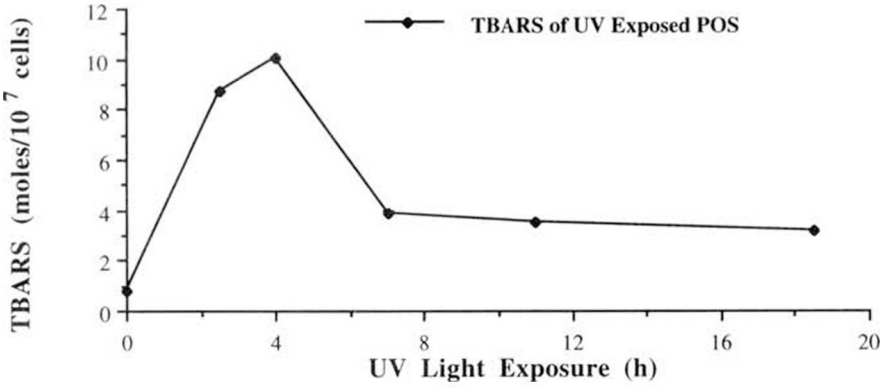

Fig. 2. TBARS of POS at various time points during exposure to UV-irradiation.

Mature (18hrs of irradiation) artificial lipofuscin that was given to the cells was thus largely devoid of reactive peroxides with possible toxic effects. Ultrastructurally, regularly arranged stacks of photoreceptor outer-segment-disk-membranes were seen in the native POS, while the peroxidized material was converted into a osmiophilic, electron-dense mass (Figure 3 A and B). Also, the fluorescence characteristics changed dramatically during irradiation. A strong yellowish, lipofuscin-type autofluorescence, not seen in the native material, developed in the UV-irradiated POS.

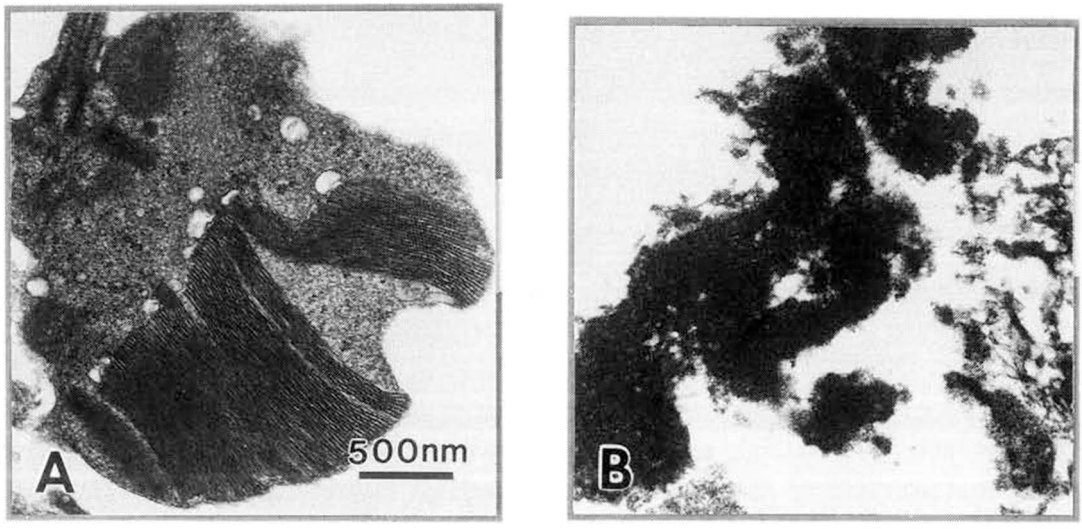

Fig. 3. Ultrastructure of native POS (A) and POS UV-irradiated for 18 hr (B).

TEM of the RPE cells showed sparse lipofuscin-like granules in cells that were kept under normal culture conditions for three weeks. Cells fed native POS for the same period of time contained somewhat more lipofuscin, while cells exposed for only seven days to peroxidized POS were packed with large lipofuscin-containing secondary lysosomes (Figure 4 A and B). These ultrastructural findings were well reflected by registred microfluorometeric values of lipofuscin-specific autofluorescence (Figure 5).

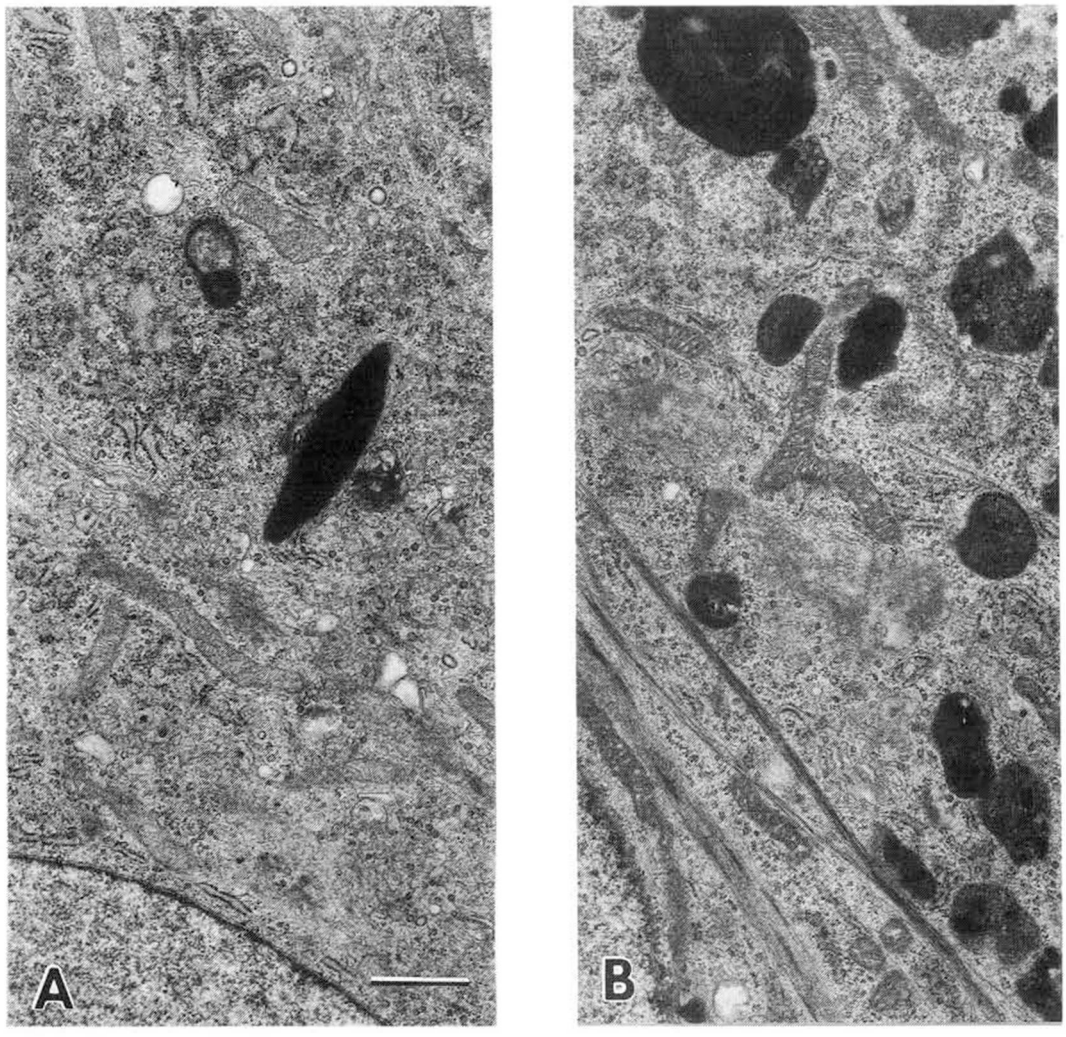

Fig. 4. *Fine structure of RPE cells after one week in culture.* ***A.*** *Control cell.* ***B.*** *Cell daily exposed to UV-irradiated POS. Bars: 1 μm.*

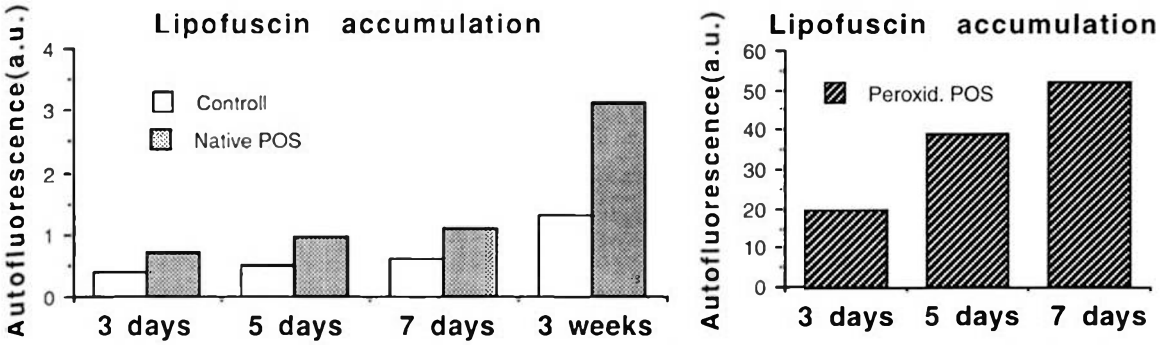

Fig. 5. *Lipofuscin-specific autofluorescence in arbitrary units (a.u.) of individual RPE cells exposed daily to native- or UV-irradiated POS for various periods of time. One hundred cells were measured in each preparation and each column represents the mean of four different experiments.*

The lipofuscin-loaded RPE cells were much more vulnerable to blue-light-irradiation than control cells grown under normal culture conditions for the same period of time. The decline in red, granular autofluorescence, indicates loss of the proton- gradient over lysosomal membranes (Figure 6A). The increase in diffuse, cytosolic green fluorescence, indicates cytosolic acidification. Irradiated cultures, returned to normal culture conditions for another 4 hrs, showed a much differing degree of cell survival depending on their load of lipofuscin. Lipofuscin-loaded cells showed a time-of-irradiation-dependent increase in mortality that far exceeded that of the likewise irradiated, but almost lipofuscin-free, control cells (Fig 6B).

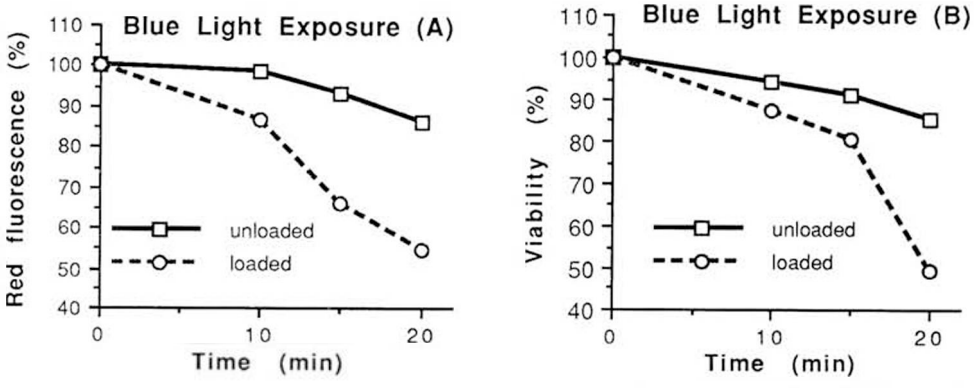

Fig. 6. A. *Change in red fluorescence of lipofuscin-loaded (exposed to UV-irradiated POS daily for seven days) and control RPE cells follwing acridine orange-vital staining secondary to blue light-exposure for indicated periods of time. Decrease in fluorescence indicates diminished granular binding of acridine orange in high concentration and is due to decreased proton-gradient over the lysosomal membranes.* ***B.*** *Viability (delayed trypan blue dye exclusion test) of blue light-irradiated, lipofuscin-loaded RPE cells previously exposed to UV-irradiated POS for seven days as compared to control cells, exposed to blue light but not to POS.*

Discussion

Retinal pigment epithelial (RPE) cells *in situ* continuously phagocytose the tips of the photoreceptor outer segments (POS) in substantial quantities [1, 2]. The phagocytosed POS contain various proteins in high concentrations and also high amounts of PUFAs [23]. Nevertheless, the material is obviously degraded in a very efficient way within the acidic vacuolar apparatus of the RPE cells. Only slowly, over decades in humans, do the RPE cells accumulate lipofuscin, or age-pigment, in substantial amounts [3]. Previously, we have shown that the slowly ongoing process of lipofuscin-accumulation within post-mitotic cells is a consequence of iron-catalyzed intralysosomal peroxidative reactions, secondary to the diffusion of hydrogen peroxide into the lysosomal apparatus [4]. Thus, one might anticipate that RPE cells have a very capable system for the elimination of intracellularly produced superoxide and hydrogen peroxide, as well as an efficient way of chelating intralysosomal redox-active low-molecular-weight iron.

Lipofuscin is not degradable by the host of hydrolytic enzymes that is contained within the lysosomal apparatus. This is quite clear from the observation that lipofuscin and lysosomal enzymes occur together within secondary lysosomes of the residual body variety, and that such organelles are integrated parts of the acidic vacuolar apparatus [24, 25]. Lipofuscin seems to be a complex polymeric substance mainly composed of Schiff-base-type material formed during reactions between protein-residues and various aldehydes. The latter, in turn, form during spontaneous fragmentation of lipid hydroperoxides [26].

It is well known that peroxidation of proteins and lipids may be accomplished by exposure to UV-light. Thus, a variety of organic materials, including POS, would be transformed into lipofuscin-like substances merely by being exposed to UV-light for a sufficient period of time. We have found that UV-light transforms POS into a material with an autofluorescence and ultrastructure akin to lipofuscin [9]. During the early phase of this process an increase of TBARS indicates formation of peroxides (Figure 1). The decline in TBARS after prolonged irradiation may indicate increasing spontaneous fragmentation of the formed peroxides, resulting in conjugation of formed aldehydes with proteins and the formation of more complex, stable Schiff-bases as well as their secondary, Amadori-type reaction products, e.g., lipofuscin. Artificial lipofuscin was taken up by the RPE cells, but not degraded, resulting in the rapid transformation of young RPE cells into aged, lipofuscin-rich ones. There was no sign of any toxic reaction in the form of early cellular degeneration, or death; indicating that mature artificial POS-derived lipofuscin did not contain large amounts of toxic hydroperoxides. Native POS were, on the contrary, much better degraded, although it must be expected that also this material had undergone some oxidation during the preparation procedures.

Our findings support our previously proposed hypothesis that lipofuscin-accumulation within RPE cells is a consequence of intralysosomal oxidative reactions converting phagocytosed POS into peroxidized lipofuscin-like material [4].

Since lipofuscin shows yellowish-orange autofluorescence when irradiated by blue light, it must contain photo-active compounds which ought to activate present oxygen into either

superoxide or singlet form [6, 7, 8]. At old age RPE-cells, especially in the macular area, may be heavily loaded with lipofuscin [3]. It is thus conceivable that visible light in such cells may induce photo-oxidative reactions that, in particular, would affect the lysosomal membranes surrounding the lipofuscin or age-pigment material.

We have here shown that lipofuscin-containing secondary lysosomes no longer retain the lysosomotropic weak base acridine orange in a normal way after irradiation with blue light, indicating that the proton gradient over the lysosomal membranes has largely vanished. In previous studies, we have demonstrated that such a loss of the proton gradient is accompanied by leakage of larger molecules, such as proteolytic enzymes, to the cell sap, resulting in cellular degeneration and eventually cell death [22].

Lysosomal hydrolytic enzymes are always present in secondary lysosomes, even if they carry large amounts of lipofuscin, showing that such secondary lysosomes, often somewhat missleading named residual bodies, are indeed integrated parts of the acidic vacuolar apparatus that, from a functional point of view, may be considered a tube- or box-like construction, which by means of fusion and fission-activities at every time-point is divided into a number of vesicles [24, 25]. The system receives newly formed lysosomal enzymes from the Golgi apparatus, through fusion with primary lysosomes, and substrates to be degraded through the hetero- and autophagocytotic pathways.

We have, in summary, shown that heavily lipofuscin-loaded RPE-cells are much more vulnerable to visible blue light than control cells. The damaging mechanism seems to be photo-oxidation of the membranes surrounding lipofuscin-loaded secondary lysosomes, where the lipofuscin may act as a photosensitizer. This seems to result in leakage of hydrolytic enzymes inducing cell degeneration and, if the damage to the lysosomal membrane is severe enough, cell death. The mechanisms described here may be of relevance to the understanding of the pathogenesis of age-related macular degeneration.

Acknowledgement

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Discussion after the talk of Dr. Brunk

Hiramatsu: Is there any difference in melanin content between the lipofuscin loaded RPE cells and the control RPE cells?

Brunk: No. RPE cells that have accumulated oxidized POS contain melanosomes in normal amounts.

Eldred: Based on our experience, I question whether the pigment that you have generated is truly like age pigment, or whether it is more like the vitamin E-deficiency pigment (i.e., ceroid). This would be a very important question to resolve. There is a very easy way to distinguish between the two. That is, to extract the pigments and do chromatography. If it is truly like normal age pigments, a characteristic pattern of fluorophores will be present. If, like the E-deficiency pigment, very little will be extractable and separable by chromatography.

Brunk: I agree. There is an obvious need for characterization of our oxidized POS material before and after its uptake by RPE cells. I hope we would be able to do that in collaboration with you!

de Gritz: Electron microscopically, in lipofuscin one can see electron dense as well as electron-lucent areas in the same granules. Did you recognize this?

Brunk: Yes, some secondary lysosomes contained material including electron-lucent globules.

Ivy: You have measured lipofuscin by autofluorescence, but you admit that you cannot morphologcally distinguish the exact nature of the bodies you are measuring. I would suggest that, since you admit that it is not known if what you call autophagic or heterophagic vacuoles have fluorescence, then you may also be overestimating the amount of lipofuscin in your RPE cells, as you have suggested that we did in our studies (this volume). I have already commented in my answer to your question following my talk that we seem to disagree on the exact definition of lipofuscin, mainly in regard to its digestibility.