Fluorescence Properties of Autofluorescent Granules Generated by Cultured Human RPE Cells

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PURPOSE. To compare the fluorescence properties of autofluorescent granules generated by retinal pigment epithelial (RPE) cells in vitro with those of the lipofuscin of RPE in vivo.

METHODS. Cultured human RPE cells were maintained in basal medium for as long as 1 year, fed rod outer segments (ROS) daily for as long as 56 days, fed ROS in the presence and absence of leupeptin, or fed liposomes consisting of the major phospholipids in ROS. At different time points, cells were examined for overall fluorescence, and their fluorescence spectra were determined. In addition, chloroform-methanol extracts were examined by thin-layer chromatography and compared with those generated from RPE lipofuscin.

RESULTS. Autofluorescent granules accumulated in cultured RPE cells, regardless of the presence of an exogenous substrate or the nature of the substrate. The rate of accumulation of autofluorescent granules was greatest in cells fed ROS. The autofluorescent material generated in cultured RPE cells had some spectral similarities with RPE lipofuscin but differed in solubility and chromatographic mobility of their constituent fluorophores.

CONCLUSIONS. The autofluorescent granules generated by cultured RPE, even with different specific substrates, differ from lipofuscin granules in vivo, suggesting that additional properties of RPE cells or of the materials they phagocytose are required to produce autofluorescent materials with the characteristics of lipofuscin. (Invest Ophthalmol Vis Sci. 1998;39:1487-1492)

Lipofuscin is a lipid-protein aggregate that is present within the human retinal pigment epithelium (RPE) as yellow-brown refractile granules showing a characteristic fluorescence under short wavelength light.1,2 Lipofuscin granules are thought to represent the lifelong accumulation of lysosomal residual bodies containing the end products of phagocytosis of photoreceptor outer segments1,3-5 and, to a lesser extent, of autophagy.1 The intracellular accumulation of these granules, which can constitute as much as 19% of RPE cytoplasmic volume in later life,6 has been implicated in the development of age-related macular degeneration.2,7

In studies in vitro5,8,9,11 and in vivo,10,13 investigators have attempted to determine the mechanisms involved in the formation of RPE lipofuscin. These studies assessed the accumulation of cellular autofluorescent granules and interpreted their presence to indicate lipofuscin formation. However, the granules were deemed comparable to lipofuscin only by virtue of their gross autofluorescence, and this property alone may not be sufficient to indicate identity with lipofuscin in vivo. For example, autofluorescent material accumulating in rats raised on vitamin E-deficient diets differed from lipofuscin in its extractability and chromatographic mobility,14 despite its similar appearance under the fluorescence microscope and fluorescence spectral profile.15 Furthermore, although the accumulation of lipofuscin-like autofluorescent granules within the rat RPE was increased by intravitreal iron injections, these iron-induced fluorophores differed in solubility from those of lipofuscin.16

Because there is an expanding body of literature on the accumulation of autofluorescent granules within RPE cells in vitro,5,8,9,17 with emphasis placed on their providing a model for lipofuscin formation, we elected to determine to what extent these fluorophores in vitro resemble those in vivo. In this study we assessed the spectral properties, solubility, and chromatographic mobility of in vitro autofluorescent granules generated by long-term maintenance of RPE cell cultures, feeding rod outer segments (ROS) to RPE cells, treating cells with the protease inhibitor leupeptin in the presence and absence of ROS, and feeding liposomes prepared from the major phospholipids present in ROS to RPE cells. The photophysical characteristics of the resultant autofluorescent granules were compared with those of lipofuscin from human donor eyes.

MATERIALS AND METHODS

Retinal Pigment Epithelial Cell Culture

Retinal pigment epithelial cells were isolated and grown as previously described by Boulton et al.18 Cells were routinely grown in Ham's F-10 nutrient mixture supplemented with 20% (vol/vol) fetal calf serum, 0.4% (wt/vol) glucose, and antibiotics in a humidified 5%CO2-95% air incubator. Purity of cell cultures was determined by staining for cytokeratin as described...
by McKechnie et al. Experiments were undertaken on confluent cultures used between passages 3 and 7, and each experiment was conducted at least twice using different cultures.

**Generation of Autofluorescent Granules by Cultured Retinal Pigment Epithelial Cells**

At confluence, the growth medium was replaced with a basal experiment was conducted at least twice using different cul-
generate in the cultures by the following approaches:

1. Maintenance of RPE cell cultures for as much as 56 days in basal medium alone.
2. Daily feeding with bovine ROS for as much as 56 days. Rod outer segments were isolated and prepared as previously described. Before use, ROS were suspended in basal medium at a concentration of 1 × 10^7 cells/ml.
3. Daily feeding with bovine ROS (1 × 10^7 cells/ml) for 28 days, and then 28 days’ exposure to basal medium alone.
4. Daily feeding with basal medium containing a final leupeptin concentration of 1 μM, 10 μM, or 100 μM, with and without ROS, for as much as 10 days.
5. Daily feeding with 1 μl/ml liposomes in basal medium. Liposomes were prepared from the major phospholipids present in ROS (phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine) by the vesicle extrusion technique. The resultant multilamellar vesicles were extruded a total of four times through two stacked 25-mm polycarbonate filters of pore size 0.1 μm, and the liposome preparation was aliquoted and stored in the dark at 4°C.
6. In a few experiments, RPE cultures were maintained for extended periods (months) undisturbed at confluence with twice-weekly feedings with minimum essential medium supplemented with 10% fetal calf serum and antibiotics.

**Analysis of Autofluorescent Material**

**Light and Fluorescence Microscopy.** Cultures were examined at various time points by light and fluorescence microscopy for the presence of intracellular, autofluorescent granules using an inverted microscope equipped with 450 nm to 490 nm excitation (BP-450-490) and 520-nm barrier (LP-520) filters (Axiovert 35; Carl Zeiss, Thornwood, NY).

**Preparation of Cultures for Analyses.** For fluorocytometric and fluorescence spectroscopic analyses, most samples were trypsinized immediately before the first feeding (0 days) and at 14-day intervals thereafter. The exceptions were the leupeptin-treated cultures, which were trypsinized 0 and 10 days after the first feeding. For analysis by thin-layer chromatography (TLC), cultures were trypsinized after 0 and 28 days for liposome-treated cells; after 0 and 10 days for leupeptin-treated cells; and after 0, 28, and 56 days for all other treatments. Cells were resuspended in phosphate-buffered saline (PBS) at 1 × 10^5 cells/ml for fluorocytometric and fluorescence spectroscopy and at 2 × 10^6 cells/ml for TLC analysis. The mean autofluorescence per population of RPE cells was determined using a fluorophotometric flow cytometer (Kalan analyzer; Becton Dickinson), based on the method of Rakoczy et al.

**Isolation of Human Retinal Pigment Epithelial Extract and Lipofuscin Granules.** Crude pigment extract and purified lipofuscin granules (when sufficient material was available) were isolated from the human RPE cells as previously described for use as standards for fluorescence spectroscopy and TLC. Cell suspensions were pooled by decade, according to donor age (i.e., 0–9, 10–19, 90–99 years) and stored at −20°C. An aliquot of the RPE extract or purified lipofuscin granules was set aside for fluorescence spectroscopy. The remaining material was extracted with chloroform-methanol as detailed later.

**Fluorescence Spectroscopy.** Fluorescence spectra were obtained from the RPE cultures, freshly isolated RPE cells, and isolated lipofuscin granules, using a Perkin-Elmer fluorescence spectrophotometer (model LS-50B; Perkin-Elmer, Norwalk, CT) equipped with FL data manager (Perkin-Elmer). In brief, duplicate 0.5-ml aliquots of the cell suspensions, RPE extract, or purified lipofuscin granules were suspended in 2-ml PBS and the spectral profile determined using an excitation wavelength of 364 nm, monitoring emission at 570 nm. In each case, slit width was 10 nm and scan speed 240 nm/min. To minimize internal quenching effects, the concentration of each solution was adjusted so that the optical density at 514 nm was less than 0.1. The resultant spectral profiles were corrected to allow for instrumental bias. Excitation spectral correction was performed automatically by the computer software and emission spectra using the quantum counter quinine sulfate (Perkin-Elmer).

To eliminate masking of fluorescence by cellular membrane strains, spectroscopy was repeated on some samples after membrane lysis. This was achieved by addition of 20 μl 10% (vol/vol) Triton X-100, prepared in distilled water, to the fluorometer cuvette. The resultant spectral profiles from cells with and without rod outer segment feeding were compared.

**Analysis of Extracted Autofluorescent Material**

**Extraction of Autofluorescent Material.** Duplicate 100-μl aliquots of cultured cells (1 × 10^7 cells/ml), freshly isolated RPE cells, isolated lipofuscin granules, ROS (1 × 10^7 cells/ml), or 1 μl/ml liposomes were extracted by addition of 250 μl 2:1 (vol/vol) chloroform:methanol containing 10 mg/ml butylated hydroxytoluene followed by 350 μl distilled water; samples were vortexed 5 seconds between additions. Phases were separated by centrifugation at 3000g for 3 minutes and isolated using a pipette. Interfacial material was re-extracted and material from each step pooled.

**Separation of Chloroform-Soluble Fluorophores.** The chloroform-soluble fluorophores extracted from the differently treated RPE cultures were compared with the fluorophores of freshly isolated RPE cells and lipofuscin by TLC. In brief, the chloroform phase of the extracted autofluorescent material was dried down under nitrogen and resuspended in 20 μl chloroform. This material was spotted onto a high-performance silica gel TLC plate equipped with a preadsorbent spotting zone (Whatman, UK). Plates were placed in a Simultan chamber (Whatman) that had been pre-equilibrated for 30
minutes with the primary solvent system described by Eldred and Katz, and were allowed to develop for 20 minutes. Plates were dried and photographed using a camera (OM-1; Olympus, Lake Success, NY) fitted with a 90-mm lens (Tamron) and 400 ASA color film (Elite) illuminated with a 366-nm light source. Ultraviolet background was minimized using a standard UV filter (Hoya) together with a Kodak Wratten 2E, Kodak CC20Y (Eastman Kodak, Rochester, NY) Lee 81EF and Lee 103 filters.

**Analysis of Extracted Material by Fluorescence Spectroscopy**

Chloroform-soluble material was dried under nitrogen, and the methanol-water and interfacial material were freeze dried. Extracts were resuspended in 2 ml PBSA and their fluorescence spectra determined as described earlier.

**Statistics**

Statistical analysis of data was performed using the Student t-test and analysis of variance.

**RESULTS**

**Analysis of Cellular Autofluorescence by Microscopy and Fluorocytometry**

The accumulation of yellow-green autofluorescent, perinuclear granules within RPE cells was eventually observed by light and fluorescence microscopy in all cultures, regardless of treatment. Fluorocytometry analyses supported the microscopic observations. The mean autofluorescence per cell increased in all cultures during the experiment, and the extent of the increase was dependent on the treatment: liposome > rod outer segment > leupeptin > control (P < 0.01).

**Analysis of Cellular Autofluorescence by Fluorescence Spectroscopy**

Changes in the excitation and emission spectra of RPE cultures fed ROS was observed throughout the experiment. On day 0, excitation spectra showed peaks at 319 nm, 389 nm, and 473 nm, whereas emission spectra demonstrated a single peak at 450 nm, and shoulders at 425 nm, 525 nm, and 570 nm. After 28 days in culture, the excitation peaks at 389 nm and 473 nm had increased in intensity and appeared broader. Emission spectra showed increases in intensity at 450 nm and the formation of a well-defined peak at 525 nm, with shoulders at 570 nm and 610 nm (Fig. 1). By day 56, the shoulders increased in intensity and an additional peak at 625 nm became apparent. Similar changes in excitation spectra were observed for cells maintained in medium devoid of ROS (compare Figs. 1A, 1B). For the emission spectra of these cultures, the increase in fluorescence intensity higher than 600 nm during prolonged incubation was much less pronounced than that observed for cells maintained in the presence of ROS. Furthermore, at each time point neither leupeptin treatment, cessation of ROS challenge, feeding with liposomes, nor absence of challenge resulted in a significant change in the fluorescence spectra when compared with cells fed ROS alone (not shown). Disruption of cell membranes by addition of Triton X-100 had no effect on the overall spectral profiles of material generated by any of the experimental treatments (not shown).

The spectra obtained from the cultured cells after different treatments showed some similarities to the fluorescence spectra of human RPE extracts and isolated lipofuscin granules. Lipofuscin granules showed excitation peaks at 355, 390, 450, and 475 nm. Peaks at 355 nm and 450 nm were absent in the human RPE extract (Fig. 2A). The excitation spectra of autofluorescent material generated in cultured cells also showed an excitation peak at 390 nm and a broad peak from 450 nm to 475 nm. Comparing the emission spectra, lipofuscin showed a characteristic broad-band emission, with peaks at 560 nm, 600 nm, and 625 nm; similar spectra were obtained for the human RPE extract, although emissions higher than 600 nm were less pronounced (Fig. 2B). Major emission peaks for the cultured cells were detected between 400 nm and 550 nm,
FIGURE 3. Comparison of chloroform-soluble fluorophores of lipofuscin from different age groups. Samples were isolated from donors aged 10 to 19 (lane 1), 20 to 29 (lane 2), 30 to 39 (lane 3), 40 to 49 (lane 4), 50 to 59 (lane 5), 60 to 69 (lane 6), 70 to 79 (lane 7), 80 to 89 (lane 8), and 90 to 99 (lane 9) years. After extraction with 2:1 chloroform:methanol, the chloroform-soluble extract was separated by thin-layer chromatography, visualizing the bands by illumination with a 366-nm UV light source. Because of limited material for donors aged 10 to 19 and 30 to 39 years (lanes 1 and 3, respectively), minimal banding was observed. but a small amount of emission higher than 625 nm was observed.

Analysis of Extracted Autofluorescent Material by Thin-Layer Chromatography

The TLC profile of autofluorescent granules generated by the different experimental treatments of cultured cells showed only blue, yellow, and green fluorophores. The number of fluorophores and their chromatographic mobilities varied slightly among experiments and with the different treatments used, but none of the fluorophores resembled the characteristic yellow-orange fluorophores of lipofuscin isolated from any of the age groups studied (Fig. 3). The banding patterns were similar in extracts of cells trypsinized on day 0 (i.e., cultured cells immediately upon reaching confluence and immediately before challenge), or in those cultured for 1 and 2 months in the presence and absence of ROS, in the presence of leupeptin or liposomes. The TLC profile from a typical experiment is shown together with a typical TLC profile of lipofuscin (Fig. 4). The color of the fluorophores and their relative fluorescence values differed only slightly between human RPE extracts and isolated lipofuscin granules.

Solubility and Analysis of Autofluorescent Granule Fluorophores

Fluorescence spectral profiles of the different fractions produced during the chloroform-methanol extraction demonstrated that the chloroform-soluble extract has a spectrum similar to those for intact granules with predominantly blue-green fluorophores (data not shown) and that most of the fluorophores appeared to be chloroform insoluble and to reside at the interface. Emission spectra of the interfacial material from samples on day 0 showed peaks at 419 nm, 440 nm, and 578 nm, whereas the spectra of such material from samples after prolonged culture showed small shifts in emission maxima toward the longer wavelengths of the spectrum (Fig. 5). Again, a small peak was evident at 625 nm in long-term cultures.

DISCUSSION

These results support those in previous studies that demonstrate the accumulation of autofluorescent material in cultured RPE cells. Such autofluorescent material has previously...
been considered lipofuscin or "lipofuscin-like" and to represent a model for lipofuscinogenesis. In the present study, we report that the autofluorescent material generated by cultured RPE cells and the lipofuscin from RPE in situ showed some spectral similarities but differed in the solubility and chromatographic mobility of their constituent fluorophores.

In cultured RPE cells, the major fluorophores emitted in the blue-green portion of the spectrum in two major peaks at 450 nm and 525 nm. The peak at 450 nm, which was seen in day 0 cultures and in cultures held at confluence, probably derived from nicotinamide coenzymes and their derivatives. The peak at 525 nm was present only in postconfluent cultures, suggesting that it originated from the autofluorescent granules. This peak had a small shoulder extending to the yellow-orange region of the spectrum at wavelengths greater than 600 nm, which is characteristic of ocular lipofuscin. Additionally, blue fluorescence has been reported in the spinal column of 3-month-old rats. In both tissues, emissions in the yellow-orange region of the spectrum increased with age at the expense of the blue-emitting fluorophores. Therefore, the presence of blue-emitting fluorophores in the autofluorescent granules of cultured cells may have indicated the presence of lipofuscin precursors that did not develop into mature lipofuscin granules, perhaps because of insufficient time or the absence of a required component in the cell culture system.

The TLC profiles of the major extracted fluorophores confirmed the spectral differences between RPE lipofuscin and the autofluorescent granules of cultured cells. Only blue, yellow, or green fluorophores were seen by TLC analysis of cultured cells, regardless of time in culture or feeding with ROS. These chloroform-soluble fluorophores of cultured cells differed from those extracted from ocular lipofuscin, which can be separated into at least 7 distinct yellow or orange bands with different relative fluorescence values.

It may be surprising that little difference in the TLC profile of cells maintained in long-term culture and those trypsinized at day 0 was seen, despite pronounced differences in their appearances under the fluorescence microscope (Fig. 1) and their spectral characteristics (Fig. 3). However, when the cells that were trypsinized at the different time points were chloroform-methanol extracted, most of the autofluorescent material present in the long-term cultured cells was insoluble and re-sided at the interface. This suggests that these fluorophores were predominantly protein-based.

The autofluorescent granules accumulating in postconfluent cultured RPE cells in the absence of ROS challenge were expected to originate primarily from autophagy, whereas the granules in cells fed ROS also had a contribution from the phagocytized materials. This dual origin was similar to the sources of lipofuscin in RPE in vivo, but the major fluorophores nonetheless differed between cells in vivo and cultured cells fed ROS. This is perhaps to be expected because a number of differences remain between the in vivo and in vitro conditions. First, whole ROS rather than spent ROS tips were ingested by the cultured RPE cells, which may have affected the ability of the RPE cells to digest this material. Second, retinas used were light adapted; therefore, any vitamin A present was more likely to be in the form of retinol rather than the more reactive retinal. It has been suggested that in vivo a buildup of retinal may favor the generation of lipofuscin fluorophores. Retinal is also highly conjugated; such structures have been suggested to account for the long wavelength fluorescence of lipofuscin. Third, it is likely that the intracellular levels of vitamin A during these experiments were well below the amounts occurring in vivo. Vitamin A is rapidly lost in RPE cells in culture, and the vitamin A cycle is disrupted. Vitamin A is a key component of lipofuscin. Fourth, the RPE cells in culture that had been propagated recently were likely to differ in many ways from their fully quiescent counterparts in situ. In this study, cells accumulated autofluorescent materials during a relatively short postconfluent period of a year. A longer postmitotic interval may be necessary for the cells to acquire the properties required to develop the fluorophores characteristic of lipofuscin, because lipofuscin takes years to accumulate in eyes.

The similarity in spectral properties, solubility, and chromatographic mobility of granules developing in cultures exposed to different substrates (i.e., ROS and liposomes and under conditions of compromised lysosomal function) suggests that neither the lipid nor the protein component of the ROS was critical for the development of autofluorescent granule fluorophores. The predominant blue-emitting fluorophores in the cell culture-derived autofluorescent granules probably consist of cross-linked products of lipid oxidation, proteins, nucleic acids, and carbohydrates. This material may contribute in some way to the overall broad fluorescence emission properties of lipofuscin. Although none of the individual treatments of cultured cells used in the present study induced the generation of materials with identical fluorescence properties to those of lipofuscin, some spectral similarities were observed. Combinations of treatments, longer periods in culture, and the addition of other key components such as vitamin A may induce cultures to produce materials more closely resembling RPE lipofuscin and provide information about its composition and genesis. Further, postconfluent RPE culture systems without exogenously delivered photoreceptor ROS offer a model to investigate autofluorescent materials derived from autophagy alone and to determine how products of autophagy in old cells affect the composition and biologic properties of RPE lipofuscin.

References


