The topography and age relationship of lipofuscin concentration in the retinal pigment epithelium

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Lipofuscin pigment granules (LPG) have been implicated as a marker of cellular aging. We have quantitated the content of LPG in human retinal pigment epithelium (RPE) as a function of age. Furthermore, topographic distribution of LPG within individual eyes was measured. Microspectrofluorometric determination of the distribution of LPG in human RPE cells revealed a progressive accumulation of LPG with increasing age. LPG first appeared in the basilar portions of RPE cells of young eyes. In older eyes, LPG formed into clumps and were noted to fill the entire RPE cell. The RPE topographic distribution of LPG revealed an increased accumulation in the posterior pole, with a consistent dip at the fovea. The ratio of lipofuscin accumulation at the posterior pole, to the total RPE, remained constant throughout life.

Key words: lipofuscin, aging, retinal pigment epithelium, ultraviolet light, microspectrofluorometry, autofluorescence, lysosomes

Although aging changes in human retinal pigment epithelium (RPE) have been shown to be numerous and varied, one striking and consistent finding in the aging human RPE is the intracellular accumulation of an autofluorescent lipopigment known as lipofuscin, the 'wear and tear' age pigment. Although the origin and cellular biochemical significance of this age pigment have not been conclusively established, the intracellular accumulation of lipofuscin appears to increase with age in nondividing or postmitotic cell populations. Whether this progressive accumulation of lipofuscin represents functional cellular decadence or merely the functional activity of a cell remains unresolved.

Various theories as to the origin of lipofuscin pigment have been reviewed by Strehler. One widely held view is that lipofuscin granules represent tertiary lysosomes or residual bodies, the indigestible end-product of phagocytosis and autophagy.

In the RPE, a well developed phagolysosomal system appears to support a high degree of phagocytosis of receptor outer segments and of autophagy. Incomplete digestion products from this lysosomal system may result in the formation of lipofuscin granules.

To clarify the relationship between aging and lipofuscin accumulation in the human RPE, we measured quantitatively, as a function of autofluorescence, the relative amount of lipofuscin in the RPE of different age
groups and determined the topographic distribution of RPE lipofuscin within individual eyes.

Materials and methods

Forty-four human eyes from 35 individuals were used in this study. They were obtained from the Massachusetts Eye Bank and the Boston Veterans Administration Hospital. Eyes with known pathological conditions were not used. Ages from a premature newborn to 88 years were represented. Eyes were fixed in formalin and cut into pupil-optic disk (p-o) sections. Under the dissecting microscope, one cut margin of each p-o section was made that bisected the optic nerve head and retinal foveal pit. After being embedded in paraffin, 10 μm sections from this same margin were dewaxed and mounted unstained in ultraviolet (UV) inert immersion oil.

Microspectrofluorometric determinations of lipofuscin content in the RPE were made with a Leitz photometer (MPV1) attached with a variable measuring diaphragm and a Zeiss microspectrophotometer with a continuous wedge transmission filter system. The Leitz photometer was equipped with an EMI photomultiplier 6094A supplied with a stabilized high-voltage power supply (Knott type MFLK). Readings were made on a Kelthley 414S picoammeter. Fluorescence measurements were performed with a xenon arc (×130, 100W; Osram) on a stabilized direct current Leitz power supply (XLS-1A-M10).

Lipofuscin, which can be characterized by its autofluorescence showing fluorescent peaks at 450 to 470 nm when excited at 365 nm, appears golden yellow when viewed under the fluorescence microscope. Multiple filter combinations were used to best match the spectral characteristics and autofluorescent properties of lipofuscin. These included the Leitz UG1 excitation filter (peak fluorescence at 390 nm with a cut off at 400 nm), the Autotechnicon 460 nm transmission bandpass filter (460 to 480 nm), and the Zeiss continuous wedge transmission filter. The MPV1 was standardized with a "control" fluorescent ZnS-CdS particle (No. 2245; U. S. Radium Corp.).

Under microscopic measurements, the circumference of all whole-eye RPE sections were determined, and an approximate 12% tissue shrinkage was calculated. The average optic disk diameter (d.d.) measured 1300 μm. In sections where an intact retinal foveal pit could be seen, the underlying RPE measured approximately 2600 μm (2 d.d.) from the temporal disk margin. This area was defined as the foveal RPE. The macular-foveal RPE covered an area of 5200 μm (4 d.d.), i.e., 2600 μm both nasal and temporal to the foveal RPE. The posterior pole was defined as the posterior 25% of the RPE, from ora to ora, which centered around the fovea.

From the optic disk margin to the nasal and temporal ciliary bodies, a photometric target area of 6.25 μm² (2.5 by 2.5 mm) was aimed at 150 different, preselected points along the total RPE of each section. In certain individual eyes in which only the posterior pole was examined, similar lipofuscin measurements were made at 90 points.

The MPV1 microfluorometer was equipped with a Ploem epi-illumination system which allowed simultaneous viewing of the retina by fluorescence and phase-contrast microscopy. The site to be measured was selected under phase microscopy and centered on the viewing field. The transmitted light was discontinued, and the site was epi-illuminated with the excitation beam; the intensity of the fluorescence was determined by allowing the photometer to range and stabilize and by obtaining a measurement within 10 sec. At each location, measurements were made at the basal portions of the RPE. RPE melanin bleaching was performed with potassium permanganate-oxalic acid. With the use of the MPV1 and the Baird-Atomic spectrophotofluorometer, solubility studies were carried out on frozen sections and on microdissected fresh and fixed ocular tissue, which included the neurosensory retina, RPE, choroid, and sclera.

Results

Spectral characteristics of lipofuscin. The golden yellow autofluorescent pigment seen
in the RPE appears to have similar characteristics to lipofuscin pigment. Lipofuscin is partially soluble in ether and methanol. The supernatant of fresh and fixed adult RPE, after 6 hr of either ether or methanol extraction, gave a distinct spectral curve, with a peak excitation at 350 to 370 nm and a peak fluorescence at 450 to 470 nm, which was similar to that of lipofuscin. These results were not seen in the xylene or formalin RPE extractions. (The formalin used in this study contained no methanol by spectrophotofluorometric analysis.)

When fluorescence determinations of fresh-frozen RPE sections were compared to either an ether or methanol extraction of these same sections for 6 hr, a notable decrease in RPE fluorescence was found. No difference was found in the 6 hr xylene- or formalin-extracted sections.

When viewed under the microspectrophotometers, the cytoplasm of infant RPE in tissue sections autofluoresced a light green. In the cytoplasm of adult RPE, golden yellow autofluorescent granules could be seen (Fig. 1) that also had spectral characteristics similar to lipofuscin; maximal excitation at 370 nm gave a peak emission of 470 to 490 nm that declined rapidly to zero at longer fluorescent wavelengths. Compared to these adult RPE granules, the spectral emission peak of the infant RPE cytoplasmic autofluorescence was shifted to the shorter wavelengths. These results suggested that the granules that appeared to autofluoresce a golden-yellow in adult RPE were lipofuscin.

**RPE autofluorescence observations.** A light green cytoplasmic autofluorescence in the fetal and infant eyes, ages 6 weeks premature to 12 months, could be seen along with apically located cellular melanin granules. At 17 months of age, occasional golden yellow autofluorescent lipofuscin granules could be recognized within the RPE. This corresponds to Streeter's observation where sudanophilic lipofuscin granules were first seen in the RPE of a 16-month-old child. At 16 years of age, these granules became strikingly prominent in basilar portions of the posterior RPE, with melanin granules still situated in more apical cellular locations. The peripheral RPE usually had fewer lipofuscin granules, and the intracellular polarity between lipofuscin and melanin granules was less apparent. From the third decade on, a general increase in the amount of lipofuscin could be seen. Lipofuscin granules began to appear as clumps. At the posterior pole RPE, lipofuscin occupied both the basilar and apical melanin portions of the cell. These observations were less marked in the periphery.

In all eyes examined, although the typical cellular golden yellow lipofuscin granules in the RPE were never seen in the pars plana or pigmented epithelium of the ciliary processes there was a minimal light green fluorescence that contributed to photometric measurements. This light green autofluorescence was
similar to that seen in the infant RPE. There were, however, isolated lipofuscin granules in the unpigmented epithelium of the ciliary processes in eyes from the later decades of life.

In order to determine reproducibility of lipofuscin measurements, repeat studies were performed on serial sections of individuals' eyes, and a coefficient of variance of 12% was established. As an internal control, whenever pairs of eyes from individuals were studied for lipofuscin content, the Mann-Whitney U test was performed, and no statistical difference between eyes could be found. Further statistical analysis of the data was made with Spearman's rank correlation and the sign test.

**Lipofuscin topographic distribution.** Relative lipofuscin content, as a function of fluorescent intensity, was plotted to scale at each of the preselected areas along the RPE. Although the RPE of infants fluoresced a light green, fluorescence was relatively minimal, and there appeared to be no distinct fluorescent pattern. However, in the RPE of older eyes where lipofuscin autofluorescence could be seen, a distinct fluorescent curve emerged (Fig. 2). In the peripheral RPE, lipofuscin content was relatively small. At the posterior pole, RPE lipofuscin content had increased strikingly. Also, at the macular-foveal region, a statistically significant dip in the RPE lipofuscin quantity in adult eyes was consistently observed (p < 0.005). This lipofuscin dip was relatively small and never fell below the lipofuscin content at the equators.

To determine the effects of melanin on lipofuscin measurements and on the dip at the macula, the RPE was bleached of melanin. Repeat measurements showed a slight,
Fig. 5. Lipofuscin content ratio (posterior pole/total RPE) plotted as a function of age.

general increase in lipofuscin fluorescence; however, the shape of the topographic distribution of lipofuscin and the dip remained essentially unchanged.

**Lipofuscin content vs. age.** Areas under each lipofuscin curve were determined with a Keuffel and Esser planometer, and age-related comparisons of these areas were made. The lipofuscin content of the RPE at the posterior pole (Fig. 3) in all 44 eyes increased with age (p < 0.001). Total RPE lipofuscin content (Fig. 4) was measured in 29 of these eyes. (We measured lipofuscin content at the posterior pole in 44 eyes, but total RPE lipofuscin was measured in only 29 of these eyes because the anterior aspect of the eye was used for other purposes in 15 eyes.) Again, it appeared that lipofuscin accumulation increased with age (p < 0.001).

**Age-related topographic changes.** In the 29 eyes in which the total lipofuscin content was measured, the ratio of posterior pole to total RPE lipofuscin remained unchanged regardless of age (Fig. 5). Apparently, the rate of RPE lipofuscin accumulation at the posterior pole is similar to that of the total RPE.

**Discussion**

Increasing intracellular accumulation of lipofuscin pigment is considered to be one of the most consistent cytological changes correlated with aging. There is an ongoing controversy as to what exactly lipofuscin is, and one theory suggests an intimate relationship between lysosomes and lipofuscin, the latter containing many of the hydrolytic enzymes present in primary lysosomes, including the lysosomal membrane "marker," n-acetylglucosaminidase. Evidence suggests that lipofuscin granules may originate from the peroxidation of unsaturated fatty acids, resulting in carbonyl compounds such as malonaldehyde. These compounds may crosslink with biological molecules, resulting in crosspolymers that may be hydrolytically indigestible by lysosomes and may accumulate intracellularly as tertiary lysosomes or residual bodies. Mann and Yates have suggested that the accumulation of lipofuscin within a cell may affect its metabolism in such a way as to cause the eventual death of that cell.

In nondividing and slowly dividing tissue, tissue retention of indigestible substances can be observed without evidence of exocytic elimination. Cellular dysfunction in these cells may result from the life-long accumulation of lipofuscin, which may cause a mechanical disruption of the cellular organization.

In the RPE, a postmitotic cell, lipofuscin accumulation may also result from incomplete lysosomal digestion. One important function of the RPE is the removal and phagocytosis of the continuously shed receptor outer segments. In addition, the long-term effects of light, oxygen, and dietary deficiencies on the RPE may increase its risk for generating free radicals. These free radicals may initiate destructive peroxidation reactions within the RPE and result in molecular crosslinkages which may require removal through autophagy. This
high degree of phagocytosis and autophagy in the RPE suggests a well-developed and active phagocytic-lysosomal system. Incomplete lysosomal digestion of phagosomes and autophagosomes may result in the formation of tertiary lysosomes and lipofuscin pigment.

Nandy observed two types of lipofuscin pigment in mice. The one found in young animals autofluoresced a greenish yellow and was scattered in the neuronal cytoplasm. The other type found in older animals, autofluoresced the traditional golden-yellow and was deposited in clumps. The greenish autofluorescence found in the RPE of infants may represent the same early precursor stage of lipofuscin described by Nandy, whereas the golden yellow autofluorescence seen in the RPE of older eyes may represent the lipofuscin found in his older animals.

Of interest is the similarity between the adult RPE topographic distribution of lipofuscin and the distribution of rods in the human retina. The retinal rod density appears to parallel the RPE lipofuscin content, even at the macular-foveal region where the lipofuscin curve dips as the rod density falls to zero. It has been shown that the RPE underlyng rod outer segments actively engulfs and destroys thousands of disks every day. Although recent evidence points to a similar cone-turnover mechanism, the rate of cone turnover may not be as great as the rate of rod turnover.

In addition, the rate of lipofuscin production in the posterior pole is similar to that of the total RPE. This uniform rate of accumulation may directly reflect the rate of terminal outer segment phagocytosis in the RPE. These findings suggest that lipofuscin production may be, in part, a consequence of RPE phagocytic-lysosomal activity on receptor outer segment phagosomes. There may be more phagolysosomal activity in the RPE underlying rods, and consequently the production and accumulation of lipofuscin may be greater in these areas.

Lipofuscin increases with age in the RPE. Whether it accumulates in a linear or sigmoidal fashion is presently unclear. Regardless, one may speculate that RPE accumulation of this age pigment may continue to a certain extent without interference to cellular function until a certain critical level is reached, at which point cellular dysfunction and death may occur. Perhaps this process may be the underlying mechanism in macular degenerative diseases of the aged.

The present technique of quantifying lipofuscin in the RPE in age-related normal eyes offers us a method to evaluate RPE in disease states. For example, we looked at an eye from a 70-year-old man with choroidal dystrophy and measured the lipofuscin content. Compared to age-related controls, the RPE periphery contained more than five times the expected amount of lipofuscin. The posterior pole RPE had already degenerated and disappeared, but one might speculate that the lipofuscin content in these RPE cells prior to dying, or bursting, was at a maximal, critical level.

In conclusion, it has been shown that the relative amount of lipofuscin content in the human RPE increased with age. Across the RPE of individual eyes, there appeared to be a distinct topographic distribution of lipofuscin, with a consistent lipofuscin dip at the macular-fovea. It is suggested that lipofuscin production and accumulation in the RPE are related to the activity of the cellular phagocytic-lysosomal system. Studies are presently underway to further clarify these relationships.

REFERENCES
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