Lipofuscin Granules in Human Photoreceptor Cells

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Twenty-four human retinas were structurally examined in order to study the degradative pathway in inner segment turnover. Lipofuscin granules were found in myoids of photoreceptor inner segments. Cone lipofuscin granules exhibited autofluorescence, in ultraviolet light. Both the cone lipofuscin granules (≈1.6 μm) and the rod ones (≈0.6 μm) were membrane-limited inclusions comprising different contents. Various vacuoles related to autophagy were found in the myoids of rods and cones. Acid phosphatase activity was demonstrated in lipofuscin granules of the rods and cones, as well as adjacent various vacuoles. A survey of the cone lipofuscin granules in the semithin Epon sections revealed that more than one-half of the eyes from humans over 30 years of age contained cone lipofuscin granules, whereas eyes from those under 30 years of age did not. These results strongly suggest that lipofuscin granules represent an accumulation of residual bodies of autophagy in the photoreceptor inner segments. Invest Ophthalmol Vis Sci 29:671-679, 1988

Materials and Methods

Materials

We studied 24 human eyes obtained from 24 Japanese subjects. Five eyes were enucleated because of an absolute glaucoma and 19 were obtained at the time of surgery for the treatment of either intraocular or adnexal tumors. The retinas were examined by light and electron microscopy. One eye was used for histochemical staining for acid phosphatase. The ages of the 24 patients ranged from 4 months to 83 years (Table 1).

Light and Electron Microscopy

The enucleated eyes were immediately prefixed with 4% glutaraldehyde buffered at pH 7.2 with 0.1 M cacodylate or phosphate. Following several washings with fresh buffer solutions, the tissues were postfixed with 1% OsO4 in the same buffer solution. After dehydration in a graded series of ethanol and clearing with propylene oxide, they were embedded in Epon 812.

For light microscopic examination, semithin Epon sections (1 μm in thickness) were cut on a Sorvall (Dupont, Newtown, CT) MT-2B ultramicrotome and stained for 1 min in 0.1% Azur-II or Toluidine blue with 1% borax at 90°C. Occurrence of cone lipo-
Table 1. Summary data on patients studied

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Lipofuscin granules were examined using retinas, the outer layers of which were free from pathological changes.

For electron microscopic examination, thin sections (100 nm in thickness) were cut on the same ultramicrotome. Doubly stained sections with uranyl acetate and lead citrate were examined under a JEM-100CX electron microscope (JEOL Ltd., Tokyo, Japan) at 80 kV. Unstained 200 nm thick sections were used for energy dispersive X-ray microanalysis with an EDDS-II (EG & G Ortec, Oak Ridge, TN) attached to a JEM-100CX at 50 kV.

Fluorescence Light Microscopy

Parts of eye cups fixed with 4% glutaraldehyde buffered with 0.1 M cacodylate were dehydrated with ethanol and cleared with xylene. Following embedding in paraffin, the tissues were sectioned into a 4–10 μm thickness. These sections were rinsed with xylene to remove the paraffin. The unstained sections were stained with Sudan-IV, the lipofuscin granules were negatively stained, while lipofuscin granules in retinal pigment epithelia were slightly stained.

Acid Phosphatase Staining

The eye of a 70-year-old man was enucleated because of a malignant melanoma of the choroid in the posterior fundus. The eye was immediately immersed in 4% glutaraldehyde buffered at pH 7.2 with 0.1 M cacodylate. After bisection, the equatorial region far from the tumor was excised and the neural retina with the retinal pigment epithelium was carefully separated from the choroid and sclera. After several rinses in 0.1 M cacodylate buffer with 11% sucrose, the tissues were immersed in medium of Gomori for 30 min at 37°C. Following several rinses in 0.05 M acetate buffer with 7.5% sucrose, these tissues were postfixed with OsO4, dehydrated, embedded in Epon and sectioned using the same procedure as described above. Thin sections were examined without further staining.

Results

Light Microscopy of Cone Lipofuscin Granules

In the light microscopic observations, cone lipofuscin granules (~1.6 μm in diameter) were frequently present at the distal regions of the myoids in the cone inner segments. The light microscopic profiles of lipofuscin granules varied depending upon fixation and staining: (1) the lipofuscin granules in tissues fixed doubly by glutaraldehyde and OsO4 appeared pale brown without staining, and the color became dark greenish blue with Azur-II or toluidine blue staining (Fig. 1a,b); (2) when tissues were fixed with glutaraldehyde alone and embedded in paraffin, the lipofuscin granules as well as those in retinal pigment epithelia exhibited a yellowish-gold autofluorescence in ultraviolet light (Fig. 2); and (3) when retinal tissues were fixed with glutaraldehyde alone, sectioned by a Cryotome (Lipshaw, Detroit, MI), and lipid-stained with Sudan-IV, the lipofuscin granules were negatively stained, while lipofuscin granules in retinal pigment epithelia were slightly stained.

Electron Microscopy of Lipofuscin Granules in Cones and Rods

In electron microscopic observations, cone lipofuscin granules were membrane-limited, electron-dense, spherical inclusions located in the periphery near the cell membrane of a cone myoid, but did not touch the cell membrane (Fig. 3). High-power electron micrographs revealed different contents: electron-dense materials, electron-lucent materials, lamellated materials and ground substance. The electron-dense materials were often composed of granular substances measuring 20–80 nm in diameter. The electron-lucent material showed a homogeneous electron density. The lamellated materials were an accumulation of membrane-like structures. The ground substance had a moderate electron density and often a fine granular appearance (Fig. 3, inset). Each cone lipofuscin granule had a different appearance due to the quantity of these contents.
In energy dispersive X-ray microanalysis, the cone lipofuscin granules revealed a high peak of osmium and no peak of other heavy metals.

Electron microscopy often revealed rod lipofuscin granules (~0.6 μm in diameter), though rarely by light microscopy because of the small size. Rod lipofuscin granules were found not only in the tips of myoids near the cell membrane but also elsewhere in the myoids and even in outer rod fibers. The contents were similar to those of the cone lipofuscin granules (Fig. 4).

Acid Phosphatase Staining

Small amounts of reaction products were observed on both the cone and rod lipofuscin granules. The reaction products were sometimes concentrated near the limiting membrane (Fig. 5a,b). The autophagic vacuoles sometimes showed the activity in spaces between the two limiting membranes (Fig. 5c). Strong activity was noted on elliptical vacuoles. A small amount of reaction products was found on the electron-lucent vacuoles, multivesicular bodies and lamellar bodies (Fig. 5d). Slight activity on a Golgi complex was found (Fig. 5b,d).

Autophagy in Rods and Cones

Various membrane-limited structures related to autophagy were found in myoids of rods and cones in all the patients, including an infant. Some autophagic vacuoles were surrounded by two membranes and filled with recognizable cytoplasmic contents: free ribosomes, membranous structures and ground plasma. Others having a single limiting membrane were frequently present at a trans-face of a Golgi complex (Fig. 6a). An elliptical vacuole had an electron-lucent lamina (ca. 30 nm thick) just inside the lamella.
Fig. 3. Electron micrographs of a tangentially sectioned retina at a level of the tips of cone myoids. Each cone has a single lipofuscin granule, while the rod sometimes has a small one (arrow). Inset: A higher magnification of a cone lipofuscin granule. It is surrounded by a membrane, comprising four different contents: electron-dense materials (D), electron-lucent materials (asterisk), lamellated materials (double arrow) and ground substance (S) (×5400, inset, ×78,000).
single limiting membrane and containing a homogeneous electron-dense substance (Fig. 6b). Electron-lucent vacuoles, multivesicular bodies and lamellar bodies were surrounded by a single or double membrane and contained various materials (Fig. 6a,b). A possible residual body was a membrane-limited vacuole containing fuzzy electron-dense materials (Fig. 6a).

Spatial and Age-Related Changes of Cone Lipofuscin Granules

In the serial sections, each cone myoid unusually contained more than one lipofuscin granule. Spatial distribution of cone cells with lipofuscin granules was not constant, but they were often grouped. Some regions exclusively contained cone cells with lipofuscin granules, whereas other regions of the same retina exclusively contained cone cells without lipofuscin granules. Hence, a quantitative analysis of lipofuscin granules could not be made.

In all three eyes in which cone lipofuscin granules were found in the fovea, several smaller lipofuscin granules were seen in single foveal cones (Fig. 7a). In the foveal center, each cone myoid often possessed more than ten granules (~0.5 μm in diameter). The clustering of smaller lipofuscin granules of foveal cones was concentrated near the cell membranes and not near the tips of the myoids. Each granule was surrounded by a limiting membrane containing contents similar to those of larger lipofuscin granules, though the quantities did vary (Fig. 7b).

Occurrences of cone lipofuscin granules were light
Fig. 5. Histochemical staining for acid phosphatase. (a) A small amount of reaction products is evident on a lipofuscin granule in a cone myoid. (b) Reaction products are present on a lipofuscin granule in a rod myoid and are also visible in a Golgi complex (G). (c) Reaction products of an autophagic vacuole are seen in the space between the two limiting membranes. (d) Reaction products densely accumulate on elliptical vacuoles (EV). A moderate amount of reaction products is visible in an electron-lucent vacuole (arrow), multivesicular body (MV), lamellar body (LB) and Golgi complex (G) (a, b, c, ×65,000 d, ×59,000).
Fig. 6. Cross-sections of cone myoids showing various stages of autophagy. (a) Autophagic vacuoles (AV) are formed near the trans-face of a Golgi complex (G). A multi-vesicular body (MV) and possible residual body (RB) are apparent. (b) A lamellar body (LB) surrounded by two membranes seems to be a degraded mitochondrion. EV: elliptical vacuole, LV: electron-lucent vacuole (a, ×88,000 b, ×43,000).
Fig. 7. Foveal cones in a sagittally sectioned retina 500 μm distant from the foveal center. (a) Each cone has several smaller lipofuscin granules in the periphery of the myoid. (b) A higher magnification of lipofuscin granules of a foveal cone. Each granule is surrounded by a membrane and is rich in lamellated materials (a, ×3,300 b, ×37,000).

Discussion

Cytoplasmic Inclusions in Photoreceptor Cells

Several types of cytoplasmic inclusions were detected in human cones but not in rods. The "large autophagic vacuole" described by Szamier and co-workers resembles the cone lipofuscin granules detected in the present study, though localization in the cone cell differs. These authors discussed this in reference to pathological changes in retinitis pigmentosa. Villegas described small organelles in human foveal cones located near the outer limiting membrane, and suggested that they were "Stage 3 melanin granules." The location and structural appearance resembled the smaller lipofuscin granules in the foveal cones in our study. The "refractile bodies" noted by Tucker appear to be the same as the cone lipofuscin granules and smaller ones in the foveal cones, because factors of age and many features are similar to findings in our study. The relation to the sexes and some ultrastructural features did differ. The smaller number of samples may be one reason why our study showed no male/female relation, and differences in the ultrastructural features may relate to autolysis.

Several authors found dense inclusions in human cones, named them differently and proposed different functions. All these inclusions, however, may be lipofuscin granules, because their structural features are similar to the cone lipofuscin granules in our study and to "lipofuscin-like granules" found in the ground squirrel.

Relationship of Lipofuscin Granules With Autophagy

Autophagy is a process during which the old organelles are segregated and digested by lysosomal en-
zymes. Indigestible materials remain as residual bodies or are exocytosed. Autophagosomes (segregation), autolysosomes (digestion) and lipofuscin granules (accumulation of residues) are common in numerous cell types. Lipofuscin granules in particular are often found in long-living cells in older individuals, which include nerve cells and the myocardium. Autophagy in photoreceptor inner segments was observed in various vertebrate animals. In the present study, we confirmed that autophagy is a physiological process, even in human photoreceptor cells, as autophagy occurred in all the eyes examined including an eye from an infant.

The lipofuscin granules in our study are no doubt an accumulation of residual bodies that result mainly from long-term autophagy, determined on the basis of the following findings:

1. Various stages of autophagy were evident near the lipofuscin granules, and weak activity of acid phosphatase was demonstrated in the lipofuscin granules as well as various structures related to autophagy.
2. Ultrastructure of the lipofuscin granules resembled findings in other tissues, especially in nerve cells.

Recently, endocytosis and degradation of the interphotoreceptor matrix by bovine photoreceptor inner segments was noted, using in vitro techniques. Some contents of lipofuscin granules may be indigestible materials originating from such heterophagy.

Physiological Significance of Photoreceptor Lipofuscin Granules

All the eyes we studied were surgically enucleated ones. The retina possessing lipofuscin granules had no retinal disease and the patients had not been on antimetabolite therapy before the enucleation. Thus, lipofuscin granules are not the result of pathologic or autolytic changes. Moreover, some retinas from aged persons, in which the lipofuscin granules were not light microscopically evident, had smaller lipofuscin granules in the rods and cones, as seen electron microscopically. Therefore, lipofuscin granules may be vastly distributed in human photoreceptors.

There is a question as to why some retinas from some old patients lack lipofuscin granules. The size of lipofuscin granules probably reflects the integrated quantity of autophagy. One interpretation is that the autophagic activity in photoreceptor cells is influenced by nutrition, exposure to light, etc. Such individual differences in lifestyle may determine the occurrence and the size of the lipofuscin granules. Another interpretation is that the residuum of autophagy is exocytosed before it reaches a large size. This aspect is currently under investigation in our laboratory.

Key words: lipofuscin granule, human retina, rod and cone, autophagy

Acknowledgments

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References