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Lipofuscin in human glaucomatous optic nerves

J.P. Fernandez de Castro [a,b], R.F. Mullins [b], A.M. Manea [c], J. Hernandez [b], T. Wallen [b], M.H. Kuehn [b,*]

[a] Department of Ophthalmology and Visual Sciences, University of Louisville, Louisville, KY, USA
[b] Department of Ophthalmology and Visual Sciences, The University of Iowa, 200 Hawkins Drive, Iowa City, IA 52242, USA
[c] Department of Pediatrics, The University of Kansas Medical Center, Kansas City, KS, USA

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ABSTRACT

Lipofuscin accumulation has been observed in a number of neurodegenerative diseases. We recently found that autofluorescent particles also occur in the aged human optic nerve. In this study we sought to determine the nature of these particles and their correlation with aging, age-related macular degeneration (AMD) and primary open angle glaucoma (POAG). Groups of eight optic nerves from patients diagnosed with primary open angle glaucoma, age-related macular degeneration, age-matched controls and four optic nerves derived from controls younger than 42 years were used for the study. All samples were fixed in paraformaldehyde and frozen frontal sections were prepared. Sections were analyzed with fluorescence microscopy, bright field microscopy, Sudan black staining and spectrofluorometry using a confocal laser scanning microscope. Sections were photographed and analyzed to establish the distribution, quantity, and size of the autofluorescent particles. Additionally, transmission electron microscopy was used to determine the ultrastructural location of the granules. On unstained sections under light microscopy granules are detectable as pale brown inclusions and are easily stained with oil-soluble dyes, such as Sudan black. Granules fluoresce when excited at all tested wavelengths but lose their fluorescence after staining with Sudan black. These particles are distributed throughout the axonal columns, but not in the septa, and appear to be located within the glia ensheathing optic nerve axons. The histologic properties of the granules seen in the optic nerve sections correspond to lipofuscin aggregates, a result of incomplete degradation of oxidized proteins. Our morphometric analyses indicate that overall the optic nerves from control, glaucoma, and AMD donors contain similar amounts of lipofuscin. However, optic nerves derived from donors with glaucoma contain lipofuscin particles that are larger than those observed in the age-matched control and AMD groups. Furthermore optic nerves from glaucoma donors display a smaller diameter than those from age-matched controls resulting in a higher concentration of lipofuscin in glaucomatous optic nerves.

1. Introduction

Lipofuscin is a pale yellow–brown lipopigment that is widely distributed throughout the animal kingdom and is a reliable morphologic marker of normal aging. Lipofuscin tends to accumulate throughout life in post-mitotic cells, such as neurons and glia, as these cell types appear to be unable degrade or exocytose this material (Goyal, 1982; Idone et al., 2008). These deposits vary in their composition but are mainly composed from degraded proteins and a variety of lipid-like materials derived from the oxidation of polyunsaturated fatty acids (Jolly et al., 2002). Lipofuscin is created when cellular waste is engulfed by autophagic vacuoles which later fuse with lysosomes in an attempt to degrade their constituents. Thus, lipofuscin particles are membrane bound and are located in the cytoplasm of cells.

Lipofuscin accumulates in multiple tissue types during aging. The age-related accumulation of lipofuscin in the retinal pigment epithelium (RPE) is striking, and this accumulation has been implicated as a major contributor in Mendelian forms of macular degeneration as well as AMD (Sparrow, 2010; Weingeist et al., 1982; Weng et al., 1999). In the optic nerve, the presence of lipofuscin has been previously noted (Dolman et al., 1980), but the extent and prevalence of lipofuscin deposition in this tissue has not been systematically evaluated. Advanced age is a very significant risk factor for the development of Primary Open Angle Glaucoma (POAG), a disease that affects the optic nerve (Coleman and Miglior, 2008). The events that lead to death of retinal ganglion cells and axonal loss in POAG are not completely understood (Kwon et al.,

* Corresponding author. Tel.: +1 319 335 9565.
E-mail address: markus-kuehn@uiowa.edu (M.H. Kuehn).

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2009), but there is little doubt that the degradation of degenerating ganglion cell axons and their myelin sheaths requires the activity of lysosomal and proteosomal systems. For these reasons we set out to determine if lipofuscin accumulation in the optic nerve is correlated to the development of POAG or AMD.

The objective of this study is to establish the presence of lipofuscin in the optic nerve, and to determine the distribution, quantity, and size of the lipofuscin particles. These findings are compared between the optic nerves of healthy young eyes, those derived from donors with AMD or glaucoma, and healthy age-matched controls.

2. Materials and methods

2.1. Human donors

All experiments conformed to the Declaration of Helsinki. Human optic nerves were obtained in collaboration with the Iowa Lions Eye Bank (Iowa City, IA) and preserved within six hours postmortem. Following consent of the donors' families medical records were obtained for all donors and reviewed for a diagnosis of primary open angle glaucoma or age related macular degeneration. In addition, young and age-matched control donors were selected who had received an eye exam within two years before death and had been found to be free of ocular disease.

2.2. Light microscopy

For light microscopy human optic nerves were fixed in 4% paraformaldehyde in phosphate buffered saline. A portion of the optic nerve, located approximately 3–5 mm posterior to the lamina cribrosa, was infiltrated with sucrose, embedded in OCT, and 7 μm thick frozen sections were collected in the frontal plane.

Sections were either stained with Sudan Black B (Fisher Biotech) or coverslipped unstained. Untreated sections were photographed under an inverted fluorescence microscope (Olympus IX81) using a 20× objective lens at excitation wavelengths of 350 nm, 488 nm and 550 nm and bright field.

2.3. Spectrofluorometry

Using a confocal laser scanning microscope (Zeiss LSM 710) the fluorescence profile of the granules from unstained sections from each group was determined. The instrument's software (Zen, 2009) was used to measure the emission intensity at increasingly higher wavelengths ranging from 421 to 724 nm on defined potions of the image containing lipofuscin particles. An excitation wavelength of 458 nm was used. A section containing autofluorescent granules in the retinal pigment epithelium (RPE) of an AMD positive donor was used to calibrate the confocal microscope settings of percentage of transmission and detector gain to avoid saturation. All other samples were measured with the same settings.

2.4. Transmission electron microscopy

Additional optic nerves were fixed in half strength Karnoksy fixative (Schneeberger-Keeley and Karnovsky, 1968) and embedded in Spur's resin. Ultrathin sections were collected on formvar coated copper slotted grids using an ultramicrotome (EM UC7; Leica Microsystems) and photographed with a digital camera on a transmission electron microscope (JEM-1230, JEOL).

2.5. Morphometric analyses

From each optic nerve sections were obtained as described above and two non-overlapping images each representing a microscopy field of 877 × 660 μm were randomly obtained at 200× magnification using a FITC filter set. Images were analyzed using ImageJ software (Abramoff et al., 2004) and the "Particle Analysis" plug – in. Initially, we selected a set of images from ten donors, representing a wide variety of lipofuscin granule density, and manually determined the number of particles. The same images were then converted to a binary format using several threshold settings. After each conversion the number of granules was determined computationally, findings were correlated with the numbers obtained manually, and the most accurate conversion threshold was determined by regression analysis (max $r^2 = 0.97$). These settings were subsequently used on all images to determine the total number of granules, their individual size, and the total autofluorescent area. The overall area of each section was determined by outlining the digital image and calculating the enclosed space. Statistical comparison between groups of donors was carried out using one way ANOVA followed by an unsigned Tukey HSD post-hoc test.

3. Results

3.1. Donors

Eight optic nerves from patients clinically diagnosed with primary open angle glaucoma (POAG), eight with age-related macular degeneration (AMD), eight age-matched controls and four young controls were used for the study. The average age of the glaucoma group is 82.8 years, 83.0 years for the AMD group, 80.9 years for the age-matched controls, and 35.0 years for the young controls.

3.2. Histology

Initial examination of untreated human optic nerve transverse sections obtained from an area located 3–5 mm posterior to the lamina cribrosa revealed the presence of numerous autofluorescent particles ranging from 2 to 7 μm^2 in size, which can be visualized at excitation wavelengths of 350 nm, 488 nm and 550 nm, consistent with the supposition that they are comprised of lipofuscin (Fig. 1). Careful examination of sections with bright field microscopy allows visual detection of these inclusions as pale brown granules. One additional characteristic of lipofuscin particles is that they readily react with oil-soluble dyes such as Sudan Black, resulting in a dark brown stain. Staining of lipofuscin with Sudan Black reduces its autofluorescence, thereby serving to additionally confirm the identity of the complex. Our findings demonstrate that the optic nerve granules are markedly sudanophilic in all donors (Fig. 2A). After staining with Sudan black autofluorescence is almost completely abolished when excitation wavelengths of 354, 488, or 594 nm are used (Fig. 2B). These data are highly indicative that the observed optic nerve granules contain lipofuscin.

Regardless of the detection method, the number of particles observed varies widely between donors. In those donors whose optic nerves contain lipofuscin it appears to be distributed throughout the entire cross section of the nerve. Particles are typically detected within the axonal columns and are largely absent from the septa or areas of glial hypertrophy.

3.3. Ultrastructural localization

The optic nerve axonal columns are comprised of myelin sheathed retinal ganglion cell axons as well as astroglia and oligodendrocytes, both of which maintain an intimate connection to the axons. In order to determine the ultrastructure and location of the granules we conducted transmission electron microscopy of the optic nerve of an 89 year old donor diagnosed with POAG. In this individual numerous dense, coarse electron dense granules were