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Esterified and unesterified cholesterol in drusen and basal deposits of eyes with age-related maculopathy

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Abstract

To address the potential for an outer segment (OS) contribution to the sub-retinal pigment epithelium (RPE) lesions of age-related maculopathy (ARM), we quantified esterified and unesterified cholesterol (EC, UC) with the sterol-specific fluorescent probe filipin in cryosections of ARM eyes. Twenty six eyes from 20 donors were preserved <5 hr after death in 4% paraformaldehyde ($n=16$) or 2.5% glutaraldehyde/1% paraformaldehyde ($n=10$). Eyes had exudative late ARM ($n=6$), geographic atrophy ($n=15$), and drusen ≥ 125 μm ($n=11$). Sections were stained with filipin for UC or were extracted and hydrolysed with cholesterol esterase before filipin staining for EC. Drusen varied in cholesterol content, with a rough correlation between EC and UC. Dome-shaped drusen contained distinctive, loosely packed UC-rich loops. In basal deposits, EC and UC were more prominent near Bruch's membrane than near the RPE. A UC-rich material was localized within the subretinal space ($n=4$). Maximum filipin fluorescence due to UC was quantified in 47 lesions (19 drusen, 24 basal deposits, and 4 sub-retinal) from 12 ARM eyes and compared to OS and inner plexiform layer (IPL) of uninvolved retina in the same sections. Relative to IPL, UC fluorescence was higher in lesions (mean $\pm$ S.D: 1.63 ± 0.69) and lower in OS (0.64 ± 0.18). If only the packing of membranes explained fluorescence intensity, then one would expect much higher intensities in membrane-rich OS than in lesions. Because the converse is true, the membranous material in lesions must be more highly enriched in cholesterol on a per unit area basis. UC in sub-RPE deposits cannot be derived *directly* from OS without considerable intracellular processing within RPE, additional cholesterol sources, or both.

Keywords: cholesterol; drusen; basal deposits; lipoproteins; Bruch's membrane; age-related maculopathy

1. Introduction

Age-related maculopathy (ARM) afflicts the elderly with an as yet obscure underlying degeneration. The early, non-neovascular form of ARM is characterized by focal and diffuse extracellular deposits of debris between the retinal pigment epithelium (RPE) and a specialized vascular intima, Bruch's membrane (BrM). These lesions are called drusen and basal deposits,[2] respectively. That drusen and BrM contain neutral lipids has been known for 40 and 15 years, respectively (Holz et al., 1994; Pauleikhoff et al., 1990; Wolter and Falls, 1962), but the origin of the lipid component is only beginning to be explored.

The cellular sources of drusen and basal deposits are under active investigation. The RPE, a supporting epithelium that transports photoreceptor nutrients and wastes and phagocytoses and processes shed photoreceptor outer segments (OS), is a leading candidate. A link between OS phagocytosis and ARM-associated extracellular lesions was formally stated by Young (Young, 1987), who postulated

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[2] 'Basal deposits' collectively refer to basal laminar and basal linear deposit. 'Sub-RPE deposits' collectively refer to drusen and basal deposits.

that engorgement of RPE with residual bodies (lipofuscin) resulting from phagocytosis could lead to stress-related basolateral secretion of abnormal basement membrane products. In contrast to this indirect route, a more direct route to extracellular lipid-rich lesions is implicit in studies showing membrane-like material in the basolateral clefts between frog RPE cells and in BrM following phagocytosis (Rungger-Brändle et al., 1987). However, Feeney-Burns et al. (Feeney-Burns et al., 1988) did not detect immunoreactivity for rhodopsin, the most abundant photoreceptor protein, in intracellular lipofuscin, BrM, or small drusen in human donors and in macaque monkeys, the only species naturally harboring these lesions. Further, Hoppe et al. (Hoppe et al., 2001), using a sucrose density gradient to subfractionate RPE-J cells following phagocytosis, observed that rhodopsin cleavage products distributed to higher density fractions over time, consistent with a transition of the enclosing organelle from a phagosome to a phagolysosome. It is now known that less abundant OS and neural retina proteins (e.g. annexins, vitronectin, crystallins) are localized in drusen (Anderson et al., 1999; Crabb et al., 2002; Deretic et al., 1994; Hageman et al., 1999; Zernii et al., 2003), leaving open the possibility that OS degradation products contribute to BrM debris. However, the fate of degradation products after RPE lysosomal processing remains unclear.

Demonstrating an OS component within drusen would support (without proving) the idea that OS are an ultimate source of this extracellular debris. Because UC, with phospholipid, is a component of all eukaryotic membranes, including OS, and drusen and deposits are UC-rich (Curcio et al., 2001; Haimovici et al., 2001; Malek et al., 2003), the hypothesis that drusen represent OS-derived debris can be tested by determining the relative concentration of UC in OS and sub-RPE lesions. OS are poor in cholesterol, with a UC/phospholipid ratio of less than 1:9 (Boesze-Battaglia et al., 1990; Fliesler and Anderson, 1983), whereas the UC/phospholipid ratio in most cell membranes are 1:3–1:2 (Dowhan and Bogdanov, 2002). Here, we utilized the fluorescent polyene antibiotic filipin, which binds specifically to the 3β-hydroxy group of sterols (Curcio et al., 2001; Norman et al., 1972) and compared the intensities of OS and sub-RPE lesions (drusen, basal deposits, and sub-retinal debris) in ARM eyes. We find that lesions are too highly enriched in UC to be accounted for exclusively by RPE-mediated pass-through of unprocessed OS membranes.

2. Methods

2.1. Human eyes

Eyes were obtained from human donors <5 hr after death. Use of human tissues was approved by institutional review at the University of Alabama at Birmingham (protocol number X900525013). Globes were preserved by immersion in either 4% paraformaldehyde ($n=16$) or 2.5% glutaraldehyde and 1% paraformaldehyde ($n=10$) in 0.1 M phosphate buffer (PB) following removal of the anterior segment. All eyes were inspected internally for macular chorioretinal pathology, which was photographed in stereo using epi- and trans-illumination and color slide film (EPJ320T, Kodak) following the removal of vitreous and submersion in buffer to mitigate specular reflections (Curcio et al., 1998). Film was scanned at 1200 dots per inch (dpi) (Umax PowerLook 1100, Magiscan 4.5).

2.2. Cryosectioning

Seven millimeters-wide samples containing retina, RPE, and choroid from the macula and periphery were cryosectioned for histochemistry. Macular samples included the fovea and the temporal half of the optic nerve head. Peripheral samples included the temporal equator and ora serrata. Samples were infiltrated in 4:1 and 2:1 30% sucrose and Histo Prep medium (Fisher) for 30 min each, frozen in -70°C isopentane, serially sectioned at 10 μm, collected in duplicate on glass slides, dried at 37°C overnight, and stored at -20°C until used.

2.3. Filipin histochemistry

Sections were brought to room temperature for 30 min. For EC detection, native UC was extracted from cryosections by two 5 min rinses in 70% ethanol, native EC was hydrolysed with cholesterol esterase (enzyme category 3.1.1.13, Boehringer Mannhein) at a concentration of 1.65 units/mL in 0.1 M potassium PB (pH 7.4) for 3 hr at 37°C, and UC newly released by EC hydrolysis was stained with filipin (5 mg filipin, Sigma, dissolved in 1 mL dimethylformamide and diluted in 100 mL PB saline). Control sections were incubated in the enzyme vehicle. For UC detection, sections were hydrated and incubated for 30 min in the filipin solution without prior extraction and hydrolysis. All sections were counterstained with Mayer's modified hematoxylin (Polyscientific, BayShore, NY).

2.4. Wide-field fluorescence microscopy

Fluorescence in filipin-stained sections was observed using an upright microscope with a 346 nm excitation filter, 460 nm barrier filter, and planapochromat (20×, NA = 0.75, and 60×, NA = 1.4) and neofluor (40×, NA = 1.4) objectives (Nikon Optiphot 2, Nikon USA, Melville, NY). Digital images were acquired with a CCD camera (SensiCam, 1280×1024 chip, Cooke Instruments, Auburn Hills, MI). All images were digitized to 12 bits with 0.18-μm/pixel resolution using IP Lab Spectrum 3.5 software (Scanalytics, Fairfax, VA). Filipin-stained sections were also photographed using ASA100 black and white film (TMax100, Kodak) and scanned at 1200 dpi (Hewlett-Packard, S10/S20 PhotoSmart scanner). Composite images