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Sub-retinal drusenoid deposits in human retina: Organization and composition

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ABSTRACT

We demonstrate histologically sub-retinal drusenoid debris in three aged human eyes, two of them affected by age-related maculopathy. By postmortem fundus examination, the lesions were drusen-like, i.e., they were pale spots apparently at the level of the retinal pigment epithelium (RPE). Light and electron microscopy revealed aggregations of membranous debris, the principal constituent of soft drusen, in the sub-retinal space. Immunohistochemistry and confocal microscopy confirmed the presence of molecules typically associated with drusen (positive for unesterified cholesterol, apoE, complement factor H, and vitronectin) without evidence for molecules associated with photoreceptors (lectin-binding disaccharide bridges and opsins), Müller cells (glial fibrillary acid protein and cellular retinal binding protein, CRALPB), or RPE (CRALPB). The fact that a drusenoid material, sharing some markers with conventional drusen, can occur on opposite faces of the RPE, suggests deranged polarity of normally highly vectorial processes for basolateral secretion from RPE, and that overproduction of secreted materials and direction of secretion are independently specified processes. In the future, drusenoid sub-retinal debris might be more frequently revealed by emerging high-resolution imaging techniques.

1. Introduction

Drusen are extracellular deposits that accumulate between the retinal pigment epithelium (RPE) basal lamina and the inner collagenous layer of Bruch's membrane in aging human eyes (Green and Enger, 1993). Drusen type, size, number, total area and/or degree of confluence are risk factors for the development of age-related maculopathy (ARM), the leading cause of irreversible blindness in people over the age of 65 years (van Leeuwen et al., 2003). Although the pathobiology of drusen is not fully understood, recent studies have identified many druse constituents such as cholesterol, apolipoproteins B and E, acute phase proteins such as vitronectin, and complement components such as factor H (Anderson et al., 2001; Crabb et al., 2002; Li et al., 2007; Li et al., 2006; Malek et al., 2003; Mullins et al., 2000).

Basal linear deposit, a diffusely distributed lesion also located external to the RPE basal lamina, contains membranous debris, defined as variably sized coils of multilamellar, uncoated membranes (Sarks et al., 2007; Sarks et al., 1980). Although often described as vesicles (i.e., closed membranes with aqueous interiors), membranous debris may represent in part the cholesterol-enriched surface of sub-optimally preserved solid lipoprotein particles (Curcio et al., 2005b), and we use the term here as a convenient descriptor. In a classic 1988 paper on geographic atrophy (Sarks et al., 1988) and in subsequent studies (Sarks et al., 2007), John and Shirley Sarks showed that membranous debris constituted the principal component of lesions in four extracellular locations (soft drusen, basal linear deposit, basal mounds, and aggregations within the sub-retinal space) and in one intracellular location (vacuoles within the RPE). Membranous debris-containing soft drusen and basal linear deposit are recognized as the specific lesions of ARM (Curcio and Millican, 1999; Green and Enger, 1993; Sarks et al., 2007), but the aggregations of membranous material between photoreceptor outer segments and apical RPE are more enigmatic, as they have been described only rarely. As originally illustrated by low magnification transmission electron microscopy (TEM) (Sarks et al., 1988), they are well organized, spherical, and homogeneous aggregations of membranous debris said to be present only where photoreceptors are also present. Their coherent morphology suggests a specific formative process rather than the consequences of post-mortem retinal detachment. More recently

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(Curcio et al., 2005a), we showed that deposits in the sub-retinal space of eyes with ARM (and importantly, also with attached retinas) were highly enriched in unesterified cholesterol labeled by filipin. These aggregations resembled drusen in the same eyes with regard to staining intensity and morphology of the stained material. Unlike drusen, however, sub-retinal aggregations contained little esterified cholesterol.

More information about sub-retinal debris (SRD) would be useful, for two reasons. First, it might elucidate some aspects of biogenesis of conventionally placed drusen. Second, it may assist the interpretation of sub-retinal findings revealed by clinical retinal imaging techniques with near-histological resolution, such as ultra-high resolution or spectral domain optical coherence tomography (Michels et al., 2008; Pieroni et al., 2006; van Velthoven et al., 2007). Herein we describe SRD within the maculae of three donor eyes, two with funduscopically-identifiable ARM and another with drusenoid spots in the fundus that were exclusively SRD confirmed by histology.

2. Methods

The Institutional Review Board at the University of Alabama at Birmingham approved use of human tissues in this study. Eyes were preserved within 6 h of donor death by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h following corneal removal and stored in 1% paraformaldehyde at 4 °C until used. Stereo color images of macula and periphery of each eye (Curcio et al., 1998) were taken with a dissecting scope (SMZ-U, Nikon Instruments Inc., Melville, NY) after complete removal of the anterior segment and vitreous using 35 mm film (EPJ320T, Kodak, Rochester, NY). The number of macular drusen was determined for each eye from images of the post-mortem fundus taken with the retina in place.

Two eyes (Case 1 and Case 2, Table 1) were prepared as described previously (Rudolf et al., 2008). Under stereomicroscopic guidance, macular RPE-capped drusen were mobilized from Bruch's membrane as part of RPE-retina sheets, placed into BEEM capsules (Electron Microscopy Sciences, Hatfield PA), and covered with a 0.75% agarose/5% sucrose solution. Blocks were trimmed, post-fixed in 1% osmium in 0.1 M sodium cacodylate buffer, 1% tannic acid (gallotannin, $C_{14}H_{10}O_9$), and 1% paraphenylenediamine (OTAP method) (Curcio et al., 2005b; Guyton and Klemp, 1988) and embedded in epoxy resin (PolyBed 812; Polysciences, Warrington, PA). One-micrometer-thick sections were cut with a Leica Ultramicrotome (Ultracut UCT, Leica Mikrosysteme AG, Vienna, Austria) and stained with 1% toluidine-O-blue. Sections were examined and photographed with a 40× plan apochromat objective on an Eclipse 80i microscope (Nikon Instruments Inc., Melville, NY). Lengths of SRD along the RPE were measured with an ocular reticule. Images were captured with a Retiga 4000R Fast digital camera and Qcapture v2.8.1 software (Qimaging, Burnaby BC, Canada).

One eye (Case 3, Table 1) was prepared for cryo-sections as described (Malek et al., 2003). The retina/RPE/choroid from a horizontal belt containing the fovea and optic nerve head and extending from the nasal to temporal ora serrata were removed from the sclera. A sample for histochemistry and immunohistochemistry that was 10 mm (nasal to temporal) by 6 mm (superior to inferior), including the fovea and the temporal half of the optic nerve head, was cut from the macula with a razor blade. The sample was cryo-protected by infiltration with successive solutions of 10%, 20%, and 30% sucrose in phosphate buffer, 4:1 30% sucrose/OCT (Histoprep, Fisher Scientific, Pittsburgh PA) solution and 2:1 30% sucrose/OCT solution for 30 min each and then frozen in liquid nitrogen. Specimens were sectioned at 10 μm (CM3000 cryostat, Leica Microsystems Inc., Bannockburn, IL). Consecutive sections were collected on gelatin-subbed slides, dried at 40–60 °C for at least 2 h, and stored at −20 °C until used.

Cryostat sections were stained with Gill's formulation #3 hematoxylin (Fisher Scientific) for histopathologic evaluation. Filipin (Sigma–Aldrich, St Louis, MO) was used to visualize unesterified and esterified cholesterol, the latter after extraction and hydrolysis, as described (Malek et al., 2003). For lectin labeling, cryostat sections were incubated with rhodamine-conjugated PNA (*Arachea hypogea* agglutinin) or WGA (*Triticum vulgaris* agglutinin), purchased from the sources listed in Table 2. Unlabeled adjacent sections were used to distinguish between lectin binding and autofluorescence. For immunofluorescence, primary antibodies were obtained from the sources indicated in Table 2. Rhodamine-conjugated secondary antibodies (donkey anti-rabbit, 1:100 and goat anti-mouse, 1:200) were obtained from Jackson Immunoresearch (West Grove, PA). Alexa 594 and 488 conjugated secondary antibodies (1:200–500) were obtained from Invitrogen (Carlsbad, CA). Negative control sections were routinely processed with each experiment and included samples incubated with an irrelevant antibody or without the primary antibody.

Sections were viewed on one of two systems for wide-field epifluorescence: (1) a Zeiss Axiophot with 10× and 40× plan apochromat objectives, 3 filter cubes (excitation, barrier, and fluorophor: 360/40–460 nm, DAPI; 480–535 nm, FITC; and 560–630 nm, Cy3), AxioCam MRm digital camera, and AxioVision v4.6 image processing software; or (2) a Nikon Optiphot2 with a 20× plan apochromat objective, 3 filter cubes (420–520 nm, filipin; 546/10–590 nm, rhodamine; and 450/490–520 nm, autofluorescence), SensiCam camera (Cooke, Auburn Hills, MI), and IPLab imaging software (BD Biosciences, Exton, PA). Sections were also examined by confocal microscopy using a Nikon Eclipse 90i equipped with 3 lasers and Metamorph v7.5 software (Molecular Devices, Sunnyvale, CA). All images of experimental and control sections were exposed at matched times on the same microscope.

Table 1
Donors and eyes

Case #[a]	Age	Gender	Histopathologic description: diagnosis	CFH[b]	HTRA1[c]
1	76	F	Large soft drusen, thick BlamD: ARM	TC	*AG*
2	91	F	Large soft drusen, thick BlamD: ARM	TC	*AA*
3	86	M	Multiple aggregations of sub-retinal debris: unknown	TC	*AG*

[a] All donors were Caucasian.
[b] Complement factor H, SNP rs1061170 (Y402H). Risk allele C is italicized.
[c] HtrA serine peptidase 1, SNP, rs11200638 (-625G/A). Risk allele A is italicized.

Table 2
Labeling of sub-retinal debris

Label	Source	Dilution	SRD
Antibodies			
GFAP	Dako	1:100	−
CRALBP	Gift from John C Saari	1:2500	−
Rhodopsin (R4D2)	Gift from Robert Molday	1:50	−
Red-green opsin	Gift from Jeremy Nathans	1:50	−
ApoE	Calbiochem	1:100	+
ApoB	Polysciences, Inc.	1:100	+/−
ApoA-I	Polysciences, Inc.	1:100	+/−
Complement Factor H	Quidel	1:200	+
Vitronectin	Santa Cruz	1:200	+
Lectins			
PNA	EY Laboratories	1:1000	−
PNA	Vector Laboratories	1:500	−
WGA	EY Laboratories	1:200	−
Lipid stains			
Filipin	Sigma	500 μg/ml	+ UC +/− EC

SRD: sub-retinal debris, UC: unesterified cholesterol, EC: esterified cholesterol, present (+), minimally present (+/−), absent (−).