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The Onion Sign in Neovascular Age-Related Macular Degeneration Represents Cholesterol Crystals

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Purpose: To investigate the frequency, natural evolution, and histologic correlates of layered, hyperreflective, subretinal pigment epithelium (sub-RPE) lines, known as the onion sign, in neovascular age-related macular degeneration (AMD).

Design: Retrospective observational cohort study and experimental laboratory study.

Participants: Two hundred thirty eyes of 150 consecutive patients with neovascular AMD and 40 human donor eyes with histopathologic diagnosis of neovascular AMD.

Methods: Spectral-domain optical coherence tomography (SD OCT), near-infrared reflectance (NIR), color fundus images, and medical charts were reviewed. Donor eyes underwent multimodal ex vivo imaging, including SD OCT, before processing for high-resolution histologic analysis.

Main Outcome Measures: Presence of layered, hyperreflective sub-RPE lines, qualitative analysis of their change in appearance over time with SD OCT, histologic correlates of these lines, and associated findings within surrounding tissues.

Results: Sixteen of 230 eyes of patients (7.0%) and 2 of 40 donor eyes (5.0%) with neovascular AMD had layered, hyperreflective sub-RPE lines on SD OCT imaging. These appeared as refractile, yellow-gray exudates on color imaging and as hyperreflective lesions on NIR. In all 16 patient eyes, the onion sign persisted in follow-up for up to 5 years, with fluctuations in the abundance of lines and association with intraretinal hyperreflective foci. Patients with the onion sign disproportionately were taking cholesterol-lowering medications ($P = 0.025$). Histologic analysis of 2 donor eyes revealed that the hyperreflective lines correlated with clefts created by extraction of cholesterol crystals during tissue processing. The fluid surrounding the crystals contained lipid, yet was distinct from oily drusen. Intraretinal hyperreflective foci correlated with intraretinal RPE and lipid-filled cells of probable monocytic origin.

Conclusions: Persistent and dynamic, the onion sign represents sub-RPE cholesterol crystal precipitation in an aqueous environment. The frequency of the onion sign in neovascular AMD in a referral practice and a pathology archive is 5% to 7%. Associations include use of cholesterol-lowering medication and intraretinal hyperreflective foci attributable to RPE cells and lipid-filled cells of monocyte origin. *Ophthalmology 2015;■:1–11 © 2015 by the American Academy of Ophthalmology.*

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The onion sign was first described by Mukkamala et al[1] as a novel spectral-domain (SD) optical coherence tomography (OCT) finding of layered hyperreflective lines beneath the retinal pigment epithelium (RPE) (the sub-RPE space) usually associated with chronic exudation from type 1 neovascularization in patients with age-related macular degeneration (AMD). Typically associated with intense signal on near-infrared reflectance (NIR) scanning laser ophthalmoscopy (SLO), the onion sign was proposed by its discoverers to represent layers of precipitated lipid amidst chronic exudation[1,2] after also considering collagen or fibrin.[1] Others authors suggested fibrovascular scarring,[3] mechanical strain on Bruch's membrane, and dystrophic calcification in drusen regression[4,5] as possible histologic correlates.

Independently, Christakopoulos et al[6] hypothesized that hyperreflective lines in the onion sign represent cholesterol crystals, because they are transparent and not associated with shadowing, like calcification. This hypothesis is credible because cholesterol crystals appear as linear, highly reflecting structures in atherosclerotic coronary artery plaques viewed by SD OCT[7] in which reflections are generated from interfaces between crystal surfaces and surrounding tissue. This designation for cardiovascular disease was validated in autopsy and endarterial specimens analyzed by ex vivo OCT and subsequent

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histologic analysis.[8,9] Tissue processing using ethanol dissolves crystals, leaving distinctive lucent clefts.[10] In human eye pathologic conditions, clefts have been reported within 1 disciform scar secondary to AMD,[11] yet not in aged and early AMD eyes, which have cholesterol-rich lipoprotein deposits in drusen and Bruch's membrane.[12]

Our goals were to document the frequency and natural history of the onion sign in AMD patients and to resolve the controversy behind the hyperreflective material with histologic analysis of 2 cases identified among archival donor eyes by ex vivo SD OCT. By confirming the cholesterol crystal hypothesis, our data reinforced the potential synergy of in vivo SD OCT, ex vivo SD OCT, and histologic analysis for molecular discovery. This information is valuable to clinicians because it aids in the understanding of pathophysiology underlying AMD as well as informing diagnosis and therapeutic decision making.

Methods

The Western Institutional Review Board and the Institutional Review Board at University of Alabama at Birmingham approved the retrospective, observational cohort study and the experimental study, respectively. Research complied with the Health Insurance Portability and Accountability Act and adhered to the tenets of the Declaration of Helsinki.

Retrospective Observational Cohort Study

Medical billing records from March 15, 2014, through September 15, 2014, were used to identify consecutive patients seen by a single physician (K.B.F.) in a vitreoretinal referral practice located in New York, New York, with a diagnosis code 362.52 for neovascular AMD in the International Statistical Classification of Diseases and Related Health Problems, 9th Revision. All patients had received anti−vascular endothelial growth factor (VEGF) therapy within the course of follow-up, although this was not an inclusion requirement. All patient medical charts, color fundus photography obtained with the TRC 50DX retinal camera (Topcon Corporation, Tokyo, Japan), SD OCT images, and simultaneous NIR SLO images with a light stimulus of 815 nm obtained with the Spectralis (Heidelberg Engineering, Heidelberg, Germany) were reviewed. The onion sign was identified as the presence of layered, hyperreflective sub-RPE bands with SD OCT imaging and correlated with findings on color fundus photography and NIR SLO imaging. Serial eye-tracked SD OCT scans were used to perform qualitative analysis of the onion sign from the time of first detection to the most recent visit. The SD OCT scanning protocol used in all eyes comprised parallel horizontal line scans over the area of interest, each scan spaced approximately 150 to 250 μm apart, with automatic real-time averaging set between 16 and 32. The abundance of lines within the onion sign was considered to be increased if the number or the lengths of hyperreflective lines increased and decreased if numbers or lengths decreased.

To investigate the possibility of an association of the onion sign with systemic hypercholesterolemia, the use of an oral cholesterol-lowering medication was recorded. The association of medication use with the occurrence of the onion sign was assessed for statistical significance using the Fisher exact test and SPSS software version 22.0 (IBM Corporation, Armonk, NY). A P value less than 0.05 was accepted as significant.

Histopathologic Study

Neovascular AMD eyes were identified through an ex vivo imaging screen of eyes accessioned for research purposes from nondiabetic white donors to the Alabama Eye Bank from 1996 through 2012. Median death-to-preservation time was 3 hours and 49 minutes (range, 40 minutes−11 hours and 40 minutes). Eyes were preserved by immersion in 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1-M phosphate buffer after anterior segment excision. After removal of vitreous, maculae were photographed in color on a stereomicroscope (SMZ-U; Nikon, Melville, NY), using 35-mm color film (1996−2005) or a digital camera (CoolPix [Nikon]; 2005−2009). For both image formats, pigmentary changes were accentuated with oblique transillumination via a dark-field base, and drusen were accentuated using epi-illumination via a ring light affixed to the objective of the dissecting scope. Eyes underwent additional multimodal ex vivo imaging, including SD OCT, when prepared for histologic analysis (2011−2013). From each globe, an 8-mm-diameter full-thickness tissue punch containing the fovea and temporal portion of the optic nerve head was removed with a trephine. This punch was held in a tissue holder (courtesy of J. Fischer, Heidelberg Engineering) mounted on a Spectralis device (Supplementary material, available at www.aaojournal.org). The holder was a closed chamber with a 60-diopter lens in the front (facing into the SD OCT instrument) and a slot to hold the tissue, with the inner limiting membrane facing forward, in the back. Using tissues stabilized in this manner, we performed a 30°×20° SD OCT volume (143 scans, 30-μm spacing, with automatic real-time set at 25) and red-free SLO. Ex vivo SD OCT differs distinctly from its in vivo counterpart, yet is interpretable with experience. Postmortem edema overall reduces contrast in the neurosensory retina and underlying choroid.[13] The RPE−Bruch's membrane band usually is visible. The 2 synaptic layers, photoreceptor ellipsoid zone, and choroidal vessels also are visible in the best-preserved specimens. Fluid is hyporeflective. Eyes also underwent NIR SLO and autofluorescence SLO with excitation wavelengths of 488 nm for lipofuscin-melanolipofuscin and 787 nm for melanosomes.

Macular tissue punches were postfixed by osmium tannic acid paraphenylenediamine to accentuate extracellular lipid and were embedded in epoxy resin (PolyBed 812; Polysciences, Warrington, PA).[14] Submicrometer-thick sections were stained with 1% toluidine blue and were reviewed and photodocumented with a ×60 oil-immersion objective (numerical aperture, 1.4) and digital camera (XC10 [Olympus, Center Valley, PA]; ×1900 viewing magnification on a monitor). Histologic sections were annotated and uploaded to the Project MACULA online digital microscope (available at project-macula.cis.uab.edu). Forty eyes of 40 donors (25 women with a mean age of 86.9±5.9 years and 15 men with a mean age of 82.2±7.3 years) had neovascular AMD, defined by fibrovascular scarring in the presence of severe RPE change plus drusen, basal linear deposits (BLinD), or both.[15] Two neovascular AMD eyes exhibiting highly hyperreflective lines in the sub-RPE compartment consistent with the onion sign were identified. We used the nomenclature of Staurenghi et al[16] for SD OCT bands and that of Zanzottera et al[17] for RPE morphologic features in histologic examination.

Results

Clinical Imaging and Associations

A cohort of 230 eyes of 150 consecutive patients (mean age, 84 years; 108 women and 42 men) with neovascular AMD was identified. Of these, 16 eyes of 15 patients (7.0%; mean age,