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Correlation of Histologic Features with In Vivo Imaging of Reticular Pseudodrusen

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Purpose: To determine the histologic and cellular correlates in the retina and retinal pigment epithelium (RPE) with the presence of optical coherence tomography-defined reticular pseudodrusen (RPD).

Design: Observation case using immunocytochemistry of an exenterated eye with immediate fixation after removal.

Participants: Two patients, one with confirmed RPD and the other with mid-peripheral drusen, underwent multimethod imaging before exenteration and immediate fixation of the posterior eyecup for high-resolution immunocytochemical analysis.

Methods: Optical coherence tomography (OCT) was compared with high-resolution immunocytochemistry using a range of cellular markers to determine changes in the RPE, photoreceptors, and gliosis.

Main Outcome Measures: Correlations of the appearance of reticular pseudodrusen on OCT and immunocytochemical analysis.

Results: Reticular pseudodrusen were deposits juxtaposed to photoreceptor outer segments extending through the outer nuclear layer and even beyond the outer limiting membrane. Deposits were rich in vitronectin, photoreceptor-associated proteins, and Iba1-immunoreactive immune cells. In contrast to conventional drusen the lipid stain Oil Red O failed to stain RPD. Cellular analysis revealed that RPD were associated with photoreceptor disruption and loss and localized gliosis. In addition, anomalies in the RPE were observed.

Conclusions: Reticular pseudodrusen represent subretinal deposits that extend through the outer nuclear layer, affect photoreceptor integrity, and are associated with retinal gliosis and RPE damage. *Ophthalmology 2016;123:1320-1331 © 2016 by the American Academy of Ophthalmology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).*

Supplemental video is available at www.aaojournal.org.

Drusen are a well-described biomarker of the early stages of age-related macular degeneration (AMD).[1] Reticular pseudodrusen (RPD) are a relatively recently described anomaly that have been associated with increasing risk of late AMD, especially geographic atrophy.[2–6] They commonly appear as discrete yellow-white dots scattered across the perifoveal region on color imaging and that have a characteristic target-like appearance on infrared imaging.[7] In addition, in some patients, RPD manifest as interconnected ribbons of material in the perifoveal region, or alternatively as small lesions scattered in more peripheral retinal regions.[7] Considerable debate remains about the nature of these lesions and their cause.

The cause of RPD remains controversial. Based on en face optical coherence tomography (OCT) and histopathologic analysis, RPD have been attributed to anatomic variations within the choroidal vasculature and loss of the middle choroidal layer in particular.[3,8–10] In contrast, dysfunction of the retinal pigment epithelium (RPE) has been attributed to the genesis of RPD because of the subretinal location of the deposits on spectral-domain (SD) OCT and the progression to geographic atrophy.[11,12]

Although a great deal has been learned in recent years using a range of imaging techniques,[13] few, if any, studies compare the histologic characteristics of retinae affected by RPD with real-time in vivo imaging. Moreover, most histopathologic studies performed to date have been limited to retinae collected after death with variable fixational artefacts (e.g., approximately 3 hours[14] and 2 hours 40 minutes[11]). The potential for postmortem artifacts to affect the interpretation of the histologic studies has led to a lack of consensus on the findings to date. A detailed analysis of the changes that occur in the retina and RPE is required to understand the possible causes of RPD and their significance for the progression of retinal pathologic features.

The central aim of this study was to characterize the histologic and cellular changes in the retina and RPE that correlate with the real-time appearance of RPD as determined on SD OCT. Our results show that in a human eye immediately fixed after exenteration, RPD are located within the outer nuclear layer and occur in regions where there are RPE disruption, localized photoreceptor anomalies, and localized retinal stress. In addition, using a range of

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immunocytochemical markers, RPD were found to contain photoreceptor outer segment proteins and immune cells and lacked labeling for lipids. These results highlight the differences between RPD and conventional drusen and suggest that RPE dysfunction is associated with the presence of RPD.

Methods

Human Subjects and Tissue Collection

The macular research group at the Centre for Eye Research Australia and the Oculoplastic Unit at the Royal Victorian Eye and Ear Hospital have an ongoing collaboration to identify patients who require their eye to be removed as part of their cancer treatment. After identifying an eligible eye, the macular researchers approach the patient to request permission to examine their eye for research purposes after its removal. Eyes identified through this collaboration underwent laser treatment with a nanosecond laser because of our interest in determining the tissue response and method of action of these lasers; the results of that investigation have already been published.[15] Laser treatment was applied 5 days before exenteration. The first patient identified and approached to request permission for us to apply laser treatment to her eye in 2013 serendipitously had retinae with extensive RPD. In 2014, a second patient was identified who was matched in age to the first patient, but who did not have RPD, and underwent nanosecond laser treatment to her eye before exenteration and served as a control for the RPD eye. Retinal regions far removed from the lasered regions were evaluated in this study. All procedures and protocols adhered to the tenets of the Declaration of Helsinki and were approved by the Human Ethics Committee of the Royal Victorian Eye and Ear Hospital, Melbourne, Australia. Written informed consent was obtained from both patients after the nature and possible consequences of the study were explained.

Patient 1 was an 86-year-old white woman with a strong family history of AMD: both her parents and sister were diagnosed with AMD, although she had never been. Apart from asthma, she had no systemic illnesses. Her only medications were aspirin 100 mg daily and nonsteroidal inhalers for asthma. In September 2013, she was diagnosed with a high-grade, poorly differentiated right lower lid sebaceous cell carcinoma that required exenteration because she had undergone multiple previous resections of a lid tumor. Just before her operation, her best-corrected visual acuity was 20/30 in both eyes. On dilated fundus examination, she had widespread RPD throughout the macula and above the arcades in both retinae, seen clinically and confirmed on imaging. There were fewer than 10 small (<63 μm) drusen visible in either eye.

Patient 2 was an 86-year-old white woman with a history of rheumatic heart disease, hypertension, and hypercholesterolemia who underwent aortic valve replacement in 2002. She was in atrial fibrillation and was taking digoxin, warfarin, and aspirin (100 mg daily) as well as atorvastatin (40 mg daily) for hypercholesterolemia. Her ocular history included herpetic scarring of the right cornea and several operations on her right lower lid for chronic entropion and trichiasis since 2002. In 2014, she required an exenteration of her right eye for an aggressive sclerosing basal cell carcinoma of her right lower lid extending into her orbit, restricting her eye movements, and causing globe displacement and diplopia. Just before her operation, her best-corrected visual acuity was 20/80 in her right eye and 20/20 in her left eye. She had scattered intermediate drusen (63−125 μm) in both eyes in the mid periphery. There was no clinical evidence or imaging evidence of RPD.

Imaging and Image Analysis

Multimethod imaging was performed on patient 1 the day before the exenteration, after the pupil was dilated with tropicamide 0.5% (Mydriacyl; Alcon, Frenchs Forrest, Australia). Color fundus images were obtained using a nonmydriatic fundus camera (Canon CR6-45NM; Canon, Saitama, Japan) and near-infrared fundus autofluorescence, and SD OCT volume scans were obtained using a Spectralis HRA+OCT device (Heidelberg Engineering, Heidelberg, Germany). Scans were performed over the central 20°×20° area, with 49 equally spaced horizontal B-scans used. Each B-scan contained 1024 A-scans and was set to average 25 frames each. The grading of color fundus photographs was performed using OptomizePro (Digital Healthcare Image Management System; Digital Healthcare Ltd, Cambridge, United Kingdom). The presence of RPD also was determined by examination of the near-infrared fundus autofluorescence and SD OCT imaging by one of the authors (R.H.G.). Because of media opacity in patient 2, it was not possible to obtain high-quality images on SD OCT.

Genetics

DNA was isolated from venous blood leukocytes and 10 ng of genomic DNA was amplified using polymerase chain reaction analysis in a multiplex reaction using Hotstart Taq polymerase (Bioline, London, United Kingdom). A MassEXTEND reaction was undertaken using the designed primers, samples were spotted onto a 384 SpectroCHIP II microarray, and genotyping was performed on the MassArray platform (Sequenom, San Diego, CA). The samples were screened for haplotype-tagging single-nucleotide polymorphisms in complement factor H (*CFH*) (Y402H, rs1061170, and rs10737680), and *ARMS2* (rs10490924).[16]

Retinal Fixation and Immunocytochemistry

During surgery, immediately after exenteration, the eyes were handed to the research staff, who dissected the globe, removing the anterior contents of the eye. The posterior eyecups then were placed into a solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 4 hours. The time between exenteration and fixation was approximately 10 minutes. Eyecups then were rinsed in 0.1 M PB and photographed. With visualization through a dissecting microscope, eyecups were dissected into pieces (approximately 4 mm^2) with a scalpel blade, whereby the exact location and eccentricity were mapped. Pieces were cryoprotected in graded sucrose solutions (10%, 20%, 30% weight/volume), snap-frozen, and stored at −80° C. Pieces of interest were thawed and embedded in Tissue-Tek OCT Compound (Tissue Tek; Sakura Finetek, Tokyo, Japan), cryosectioned (14 μm sections), and collected on polysine glass adhesion slides (Thermo Scientific, Scoresby, Australia). Slides were stored at −80° C until required.

Immunocytochemical labeling was performed on cryostat sections using the indirect fluorescence method as described previously.[17] Briefly, after thawing and rinsing (3 × 10 minutes in 0.1 M PB), primary antisera, diluted in a buffer containing 3% normal goat serum, 1% bovine serum albumin (BSA), and 0.5% Triton X-100 in 0.1 M PB, were applied to sections overnight at room temperature. For retinal flat mounts, the retina was peeled off the RPE and incubated for 3 to 6 days free floating at 4° C. For RPE flat mounts, the choroid and attached RPE were removed carefully from the underlying sclera. The choroid—RPE sheets were incubated (free floating) for 3 nights at 4° C.

The primary antibodies that were used in this study included a marker of Müller cell change, rabbit anti−glial fibrillary acidic protein (GFAP; Dako, Carpinteria, CA; catalog no., Z0334; diluted 1:20,000), marker for either short-wavelength or medium- to