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Cellular Characterization of Optical Coherence Tomography and Outer Retinal Bands Using Specific Immunohistochemistry Markers and Clinical Implications

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Purpose: Optical coherence tomography has been a technological breakthrough in the diagnosis, treatment, and follow-up of many ocular diseases, especially retinal and neuro-ophthalmologic pathologic conditions. Until now, several controversies have arisen over the specific cell types that the bands observed in the OCT represent, especially over the 4 outer retinal bands.

Design: To correlate the 4 outer hyperreflective bands observed in the OCT with the histologic structures using human retinal sections and immunocytochemistry at the fovea level.

Participants: Eyes from human donors.

Methods: Vertical cryosections of human retinas were immunostained with antibodies specific for cones photoreceptors, bipolar cells, mitochondria, Müller cells, and retinal pigment epithelium (RPE) cells and were visualized using confocal microscopy.

Main Outcome Measures: Morphological correlation between histology and OCT at the fovea level.

Results: Triple immunolabeling allowed distinguishing between cells types and different cell compartments. Immunostaining with guanine nucleotide-binding protein β 3 (GNB3) and cellular retinaldehyde-binding protein (CRALBP) antibodies showed all retinal layers at the foveola, especially the separation between the outer nuclear layer and the Henle fiber layer. CRALBP and cytochrome C (Cyt C) immunolabeling revealed that hyperreflective bands 1 and 2, observed in the OCT, correspond to the outer limiting membrane and the cone ellipsoids, respectively, separated by the cone myoids. CRALBP, cytochrome C, and GNB3 showed that the RPE interdigitations extend along the entire external segment of the cones, we do not believe them to be the structure responsible for forming the third band. However, the identification of small fragments of cone outer segments within the RPE led us to characterize the third band as the cone phagosomes located in the top of the RPE. Finally, we propose that the fourth band corresponds to the accumulation of mitochondria at the basal portion of the RPE, as identified by cytochrome C immunoreactivity, and that the hyporeflective band between bands 3 and 4 corresponds to the RPE nuclei and melanosomes zone.

Conclusions: This study proposes a new interpretation of the outer retinal bands that leads to a more accurate interpretation of OCT images, providing information about the health of cones and their relationship with the RPE, and could help to form a better understanding of retinal disease diagnosis and prognosis. *Ophthalmology 2017;■:1–16 © 2017 by the American Academy of Ophthalmology*

The emergence of OCT has meant a technological breakthrough in the diagnosis, treatment, and follow-up of many ocular diseases, especially retinal and neuro-ophthalmologic ones. Before the clinical incorporation of OCT, the diagnosis of retinal diseases largely was based on the observation of retinal fundus changes or alterations in retinal vascular angiography. Optical coherence tomography has allowed visualizing of the different retinal layers and their thickness in both macula and optic nerve locations. Optical coherence tomography can be used to study the loss or changes in photoreceptor compartments, edema, and cysts. It allows an evaluation of the vitreoretinal junction, the persistence of subretinal fluid after retinal detachment or macular hole surgery, or the presence of hyperreflective foci in vascular diseases. The low resolution of the original OCT technology did not allow us to distinguish retinal microscopical structures. The improvement of the technology with the arrival of spectral-domain OCT or swept-source OCT, with better resolution power, provides the opportunity to observe in vivo all the retinal layers and some cellular compartments in an image composed of several hyporeflective and hyperreflective lines, including 4 hyperreflective bands in the outer retina at the fovea level. The histologic knowledge of the retina is crucial to understand images obtained from OCT examination. The analysis of the 4 outer hyperreflective bands at the fovea could inform us about the health and integrity of the cone compartments and their relationship with the retinal pigment

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epithelium (RPE). Healthy foveal cones are essential to provide good visual acuity, and the state of their ellipsoids and their outer segments (OSs) and their interaction with the RPE can be an indicator of how healthy cones are.[1] Therefore, OCT images are useful to evaluate cell damage in retinal diseases and to establish a correct diagnosis and prognosis. Changes of the hyperreflective or hyporeflective line thickness may have important clinical implications, because visual function was found to correlate closely with band integrity after surgical or medical treatment,[2–7] and the recovery of outer retinal OCT bands at the fovea has been correlated with the increase of visual acuity in different retinal pathologic characteristics.[8] The rescue of the retinal layers after surgery or different medications may indicate the response to treatment and potential visual restoration, because it is a sign of how healthy the retinal cells are. There is an agreement on the interpretation of the main bands observed on OCT; however over time, the 4 hyperreflective lines on the outer retina in the fovea have generated various interpretations. These 4 bands represent different portions of cone photoreceptors and the RPE at the fovea.

Based on a theoretical-bibliographic model, in 2011 Spaide and Curcio[9] proposed an anatomic correlation of the 4 hyperreflective bands in the outer retina. They analyzed histologic information from previously published studies to create a scale model drawing. According to their model, the first layer corresponded to the external limiting membrane (ELM), the second one was aligned with the ellipsoids of photoreceptor inner segments (ISs), the third corresponded to the extensions of the retinal RPE cells surrounding the photoreceptors OS discs, and the fourth one matched the RPE. With the purpose of unifying the terminology, an international panel of OCT experts was appointed and a nomenclature for the basic anatomic landmarks seen in normal OCT images was proposed. They reached a consensus after an open discussion, and currently, this nomenclature is the most accepted worldwide.[10] However, discrepancies between the naming of the hyperreflective bands of the outer retina persist. As an example, the term *IS/OS junction* for the second band is still used in the bibliography. Spaide and Curcio[9] indicated that this layer corresponds to the ellipsoids, but in 2014, Jonnal et al,[11] using adaptive optics OCT images, concluded that assignment of the second outer retinal band to the IS ellipsoid was unjustified and proposed a return to the IS/OS terminology. This issue was discussed further in a letter to the editor.[12] Other authors describe the third band as the Verhoeff's membrane,[13] instead of the RPE projections around OS, confirming the lack of consensus about the nomenclature of the outer hyperreflective bands. To clarify these interpretation discrepancies, several authors claim that better histologic studies are needed to elucidate the correlation between retinal anatomic features and OCT images.[8,9,11]

After revisiting the recent literature about the 4 OCT outer bands, some unresolved questions arise: (1) Does the second band correspond to the cone ellipsoids or to the IS/OS boundary? (2) Does the third band represents the interdigitation zone of the apical processes of the RPE, or does it correspond to the cone OS tips (COSTs)? (3) If the third hyperreflective line corresponds to the COST, why are not the rest of the OS discs hyperreflective? (4) If the third hyperreflective band corresponds to the COST and the fourth is the RPE, what is the anatomic correlation of the hyporeflective band found between the third and fourth bands?

To answer these questions, we investigated the correlation of the 4 hyperreflective bands in the outer retina with immunocytochemical techniques using specific markers for cells or cell organelles that could justify this light hyperreflection in OCT images. To our knowledge, there is not a detailed description using both techniques in the human retina. The aim of this study was to correlate the hyperreflective bands observed in the OCT at the fovea level with the retinal histologic structures using immunocytochemistry.

Methods

Human retina samples were obtained from anonymous donors without known ocular pathologic features between 40 and 60 years of age. Informed consent was obtained from all persons before participation in this study. All donations were performed in accordance with relevant guidelines and regulations. All procedures were approved by the institutional review board/ethics committee of the University of Alicante and were conducted in accordance with the tenets of the Declaration of Helsinki for experiments involving humans.

Eyes were enucleated 4 to 6 hours after death and immediately fixed in 4% paraformaldehyde for 2 hours at room temperature. After washing with 0.1 M sodium phosphate buffer (pH 7.4), they were cryoprotected by immersion in increasing concentrations of sucrose until reaching the 30% sucrose. Eyeballs were dissected into 8 portions according to the anatomic axes and were frozen. The central area containing the fovea and the optic nerve head was cut in a cryostat, and transverse sections of 14-μm thickness were obtained. Sections were examined carefully looking for the macula, and those where the foveola was present were selected and immunostained.

Triple immunohistochemistry was performed using combinations of the following primary antibodies, which have been used in several previous studies[14,15] and are characterized widely regarding cell type specificity: polyclonal rabbit anti−guanine nucleotide-binding protein β 3 (GNB3) at 1:50 dilution (Sigma-Aldrich Corp., St. Louis, MO),[16] monoclonal mouse anticone arrestin at 1:200 dilution (Dr. MacLeish, Morehouse School of Medicine, Atlanta, GA), monoclonal mouse anti−cytochrome C (Cyt C) clone 6H2.B4 diluted at 1:1000 (Zymed Laboratories, San Francisco, CA),[17] rabbit polyclonal anti−cellular retinaldehyde-binding protein (CRALBP) at 1:50 dilution (Dr. Saari, University of Washington, Seattle, WA),[14,18] and mouse monoclonal anti-CRALBP at 1:100 dilution (Abcam, Cambridge, United Kingdom).[19,20] Primary antibodies were incubated over night at room temperature at the stated dilutions in 0.1 M sodium phosphate buffer (pH 7.4), 0.5% Triton X-100. Thereafter, samples were washed and incubated for 1 hour at room temperature with Alexa Fluor 488 donkey antirabbit immunoglobulin G (IgG), Alexa Fluor 555 donkey antirabbit IgG, Alexa Fluor 488 donkey antimouse IgG, Alexa Fluor 555 donkey antimouse IgG, or Alexa Fluor 633 donkey antimouse IgG secondary antibodies from Molecular