Inner Segment Remodeling and Mitochondrial Translocation in Cone Photoreceptors in Age-Related Macular Degeneration With Outer Retinal Tubulation

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PURPOSE. To quantify impressions of mitochondrial translocation in degenerating cones and to determine the nature of accumulated material in the subretinal space with apparent inner segment (IS)-like features by examining cone IS ultrastructure.

METHODS. Human donor eyes with advanced age-related macular degeneration (AMD) were screened for outer retinal tubulation (ORT) in macula-wide, high-resolution digital sections. Degenerating cones inside ORT (ORT cones) and outside ORT (non-ORT cones) from AMD eyes and unaffected cones in age-matched control eyes were imaged using transmission electron microscopy. The distances of mitochondria to the external limiting membrane (ELM), cone IS length, and cone IS width at the ELM were measured.

RESULTS. Outer retinal tubulation and non-ORT cones lose outer segments (OS), followed by shortening of IS and mitochondria. In non-ORT cones, IS broaden. Outer retinal tubulation and non-ORT cone IS myoids become undetectable due to mitochondria redistribution toward the nucleus. Some ORT cones were found lacking IS and containing mitochondria in the outer fiber (between soma and ELM). Unlike long, thin IS mitochondria in control cones, ORT and non-ORT IS mitochondria are ovoid or reniform. Shed IS, some containing mitochondria, were found in the subretinal space.

CONCLUSIONS. In AMD, macula cones exhibit loss of detectable myoid due to IS shortening in addition to OS loss, as described. Mitochondria shrink and translocate toward the nucleus. As reflectivity sources, translocating mitochondria may be detectable using in vivo imaging to monitor photoreceptor degeneration in retinal disorders. These results improve the knowledge basis for interpreting high-resolution clinical retinal imaging.

Keywords: age-related macular degeneration, cones, ellipsoid, histology, Müller cells, myoid, outer retinal tubulation, photoreceptors, transmission electron microscopy

The most apparent clinical and histological pathology of age-related macular degeneration (AMD) occurs in the retinal pigment epithelium (RPE) and Bruch's membrane and in the adjacent subretinal space and choroid, yet ultimately, vision is lost due to degeneration and death of photoreceptors. To date, histological studies of AMD eyes have demonstrated that photoreceptors have shortened outer segments (OS) and inner segments (IS),¹ redistributed cone opsin,² and stress protein expression; cells dwindle in number over drusen³ and undergo apoptosis.⁴ A gliotic formation of cone photoreceptors and Müller cells in interconnecting tubes, known as outer retinal tubulation (ORT), was originally seen over disciform scars.⁵ With advances in high-resolution imaging such as spectral-domain optical coherence tomography (SD-OCT), in vivo retinal imaging is revealing new insights into photoreceptor degeneration in AMD and other retinal disorders. As visualized by SD-OCT, ORT are branching tubular structures in the outer nuclear layer that appear as hyporeflexive areas surrounded by a hyperreflective border that corresponds to the IS and external limiting membrane (ELM).^{6,7} Adaptive optics-assisted retinal

imaging, such as adaptive optics scanning laser ophthalmoscopy (AOSLO),⁸ has been used to demonstrate changes in cone spacing and cone reflectivity in inherited retinal diseases,⁹ geographic atrophy of AMD,¹⁰ and association with subretinal drusenoid deposits (SDD).¹¹

Photoreceptor reflectivity captured by high-resolution imaging devices can originate from scattering at refractive index boundaries and from waveguiding.^{12,13} Photoreceptors have unique tightly bundled IS mitochondria comprising 75% of normal IS ellipsoid (ISel) volume.¹⁴ In addition to efficiently using high oxygen levels of choroidal origin for phototransduction, mitochondria possibly contribute to IS optical properties, because they are light scatterers.^{13,14} In SD-OCT, the second of four outer retinal hyperreflective bands, called the ellipsoid zone, has been attributed in part to mitochondria of the photoreceptor ISel,¹⁵ yet this attribution is debated.¹⁶ Mitochondrial morphology is dynamically controlled to respond to energy needs and environmental stimuli. Refractivity of isolated mitochondria is affected by their metabolic state, at least in vitro.¹⁷

The significance of reflectivity changes in high-resolution imaging may be elucidated with new information afforded by the higher magnification and resolution provided by histological and ultrastructural investigation. For high-resolution imaging such as AOSLO to achieve maximal utility for photoreceptor structure in AMD progression, it is important to understand the photoreceptor ultrastructure, including mitochondria, which may contribute to hyperreflectivity in various retinal imaging modalities. Histology of AMD is particularly possible, because this disease is widely prevalent among the elderly.¹⁸ Many short postmortem eyes were screened for advanced AMD and processed for histology in creating Project MACULA, an online digital microscope (<http://projectmacula.cis.uab.edu/>). Although many studies describe photoreceptor degeneration in AMD (see Supplementary Table S1),^{1-5,19-22} none extensively describe cone degeneration with a focus either on the IS or advanced AMD. The purposes of this study were to survey cone degeneration in late AMD, to examine cone IS ultrastructure with transmission electron microscopy (TEM) in order to quantify impressions of mitochondrial translocation in degenerating cones, and to assess the identity of accumulated material with apparent IS-like features in the subretinal space. These observations will contribute to knowledge of the ultrastructural and molecular basis of clinical imaging.

METHODS

Eyes

This research adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board at the University of Alabama at Birmingham. Human donor eyes with gross macular appearance consistent with AMD were obtained from the Alabama Eye Bank for research purposes, with a median death-to-preservation time of 2 hours 40 minutes. Eyes were preserved in 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer after anterior segment removal and were stored in this fixative until used. Tissue was postfixed by osmium tannic acid paraphenylenediamine to accentuate extracellular neutral lipids^{23,24} and embedded in epoxy resin (Polybed 812; Polysciences, Warrington, PA, USA). Macula-wide, 0.8 μ m-thick horizontally oriented sections^{25,26} through the foveola and at 2 mm superior to the foveal center (near the rod peak²⁷) were stained with toluidine blue.

Age-related macular degeneration case ascertainment was made using published histological criteria.^{26,28,29} Macula-wide sections were imaged with a 60 $\times$ oil-immersion objective (numerical aperture = 1.4) and a digital camera (model XC10; Olympus, Tokyo, Japan). Sections were reviewed in a systematic and unbiased manner.²⁶ Scanned histological sections are available online at <http://projectmacula.cis.uab.edu/>. In late AMD eyes (40 exudative AMD, 13 geographic atrophy [GA]) from nondiabetic Caucasian donors, 77 ORT were identified in macula-wide high-resolution sections as described below. Eyes containing apparent, well-preserved mitochondria were selected for further investigation. Cones in 25 eyes (18 exudative AMD, 1 GA, 1 RPE hyperplasia of uncertain origin; 84.4 ± 5.2 years of age; 12 females, 8 males) and 5 eyes with unremarkable pathology serving as controls (86.0 ± 3.7 years of age; 4 females, 1 male) were imaged by TEM.

Imaging by Transmission Electron Microscopy

Eyes were sectioned at silver-gold thickness and viewed with a model 1200 EXII electron microscope (JEOL, Peabody, MA, USA) and imaged with an AMTXR-40 camera (Advanced

Microscopy Techniques, Danvers, MA, USA). Here, we report single-section TEM and deduced the three-dimensional organelle shape of mitochondria (i.e., circular cross-sections were deduced as having derived from spherical mitochondria). Not all ORT selected by light microscopy were imaged, because only one 2-mm region of interest in each 8-mm section fit on a TEM grid. Any ORT in that region of interest was imaged even if not the original identified ORT of interest. By en face OCT, ORT are interconnected, suggesting that they will have a unified and smoothly transitioning microscopic luminal structure.^{6,30}

Cones selected for TEM imaging contained nucleated cell bodies continuous with the IS through the ELM. Cones located in ORT were considered ORT cones (45 ORT in 12 eyes) for the purposes of analysis. Degenerating cones located outside an ORT and within the remaining mosaic where the ELM was continuous and flat were considered non-ORT cones. Material with IS-like properties located in the subretinal space was designated as Shed IS for reasons provided in the Results. All images were composited with adjustments for exposure, contrast, and background correction only (Photoshop versions CS5 Extended and CS6; Adobe Systems, San Francisco, CA, USA).

Measurements

Measurements from ImageJ software (<http://imagej.nih.gov/ij/>; provided by the National Institutes of Health, Bethesda, MD, USA) are illustrated in Figure 1. Mitochondria distance (Mito distance) was defined as the average distance from the centroid of each mitochondrial cross-section to the ELM for each cone. Mitochondria in the IS were assigned positive distances, and mitochondria internal to the ELM in the outer fiber³¹ and near the nucleus were assigned negative distances, relative to the ELM. Cone IS length was measured from the ELM to the apex of the IS near the OS. Cone width was measured at the ELM. Cone IS myoid (ISmy) length was measured from the ELM to the top of the mitochondria. Cone ISmy was measured in Control cones from unremarkable eyes only, because it was not detectable in every degenerating cone, due to mitochondrial redistribution, as shown in the Results.

Statistical Analysis

One-way analysis of variance (ANOVA) was performed between groups of cones (ORT, non-ORT, Controls) for each of the measurements to test whether at least one mean was different at the α significance of <0.05 , under the null hypothesis that all cones had the same dimensions. Post hoc Tukey's Honestly Significant Difference test was performed to determine which group means were statistically significant. Statistical analysis was performed using Matlab software (MathWorks, Natick, MA, USA).

RESULTS

Comparison of Unaffected Cones and Degenerating Cones

For reference, a normal cone from a healthy macula is shown with a long IS containing mitochondria in the ISel that are thin and tightly packed (Fig. 2A). The ISmy is located internal to the ISel. Degenerating non-ORT cones have an IS that is shortened and broadened, with ovoid mitochondria (Fig. 2B). Degenerating cones in mature phase⁷ of a closed ORT likewise have shrinking IS (Fig. 2C). Degenerating ORT and non-ORT cones with varying IS lengths were imaged at different stages of

FIGURE 1. Cone with ultrastructure measurements illustrated. Cone width was measured at the ELM (yellow line). Mito distance is the average distance from the centroid of each mitochondrion in the IS to the ELM in each cone (green line). Cone IS length was measured from the ELM to the apex of the IS (blue line). Cone ISmy was measured from the ELM to the top of the ISel and was measured only in Control cones as shown here (orange line). Photoreceptor inner segment myoid was not measurable in degenerating cones. Image shows the same cone as Figure 2A. ONL, outer nuclear layer; ELM, external limiting membrane; ISmy, photoreceptor inner segment myoid; ISel, photoreceptor inner segment ellipsoid; OS, photoreceptor outer segment.

degeneration but contained mitochondria with similar morphology.

Degenerating ORT Cones

Degenerating cones with long IS (Fig. 3A) contained spherical mitochondria (Fig. 3A, inset) that had not moved internal to the ELM toward the nucleus. During the degeneration process, ORT cone nuclei retract from the ELM, resulting in an elongated outer fiber (Fig. 3B). In these cones, mitochondria are small, with some internal to the ELM, resulting in a lack of visible myoid (Fig. 3B, inset). In addition, in a mature phase⁷ ORT, cones with different IS lengths can be found next to each other (Fig. 3C). Again, these degenerating ORT cones contain small and spherical mitochondria (Fig. 3C, inset). In another ORT, cones were found on the scleral side with stubby IS pointed into the lumen, which contains Shed IS (described below) (Fig. 3D). In addition to spherical mitochondria, some cones also contain heterogeneous electron-dense inclusions considered to be lipofuscin, previously described in normal cone photoreceptors.^{32–35} An example of ORT at a mature phase⁷ is shown in Figure 3E for reference.

Degenerating Non-ORT Cones

Non-ORT cones as defined in the Methods were imaged at various stages of degeneration. Degenerating non-ORT cones with long IS have small, ovoid mitochondria that have moved in the direction of the ELM, leaving the IS apex devoid of any visible organelles (Fig. 4A). In contrast, despite having a shortened IS, the cone in Figure 4B still has a remnant OS

FIGURE 2. Comparison of unaffected cones, degenerating non-ORT cones, and degenerating ORT cones. Neovascular AMD, except where noted. Green arrows indicate ELM, and red arrowheads indicate mitochondria in all panels. (A) Photoreceptors at 2.5 mm temporal to the foveal center in a healthy macula. Mitochondria are thin and tightly packed in the ISel (inset, magnified box). Eighty-five-year-old female donor. (B) Degenerating non-ORT cone at 3 mm from the foveal center. Mitochondria are spherical, ovoid, or reniform (inset, magnified box). Eighty-seven-year-old female donor. (C) Mature closed ORT containing cones with shrinking inner segments at 1.2 mm temporal from the foveal center. The ELM delimits the ORT lumen. Bruch's membrane (blue arrowheads). Seventy-nine-year-old male donor. ELM, external limiting membrane; ISel, photoreceptor inner segment ellipsoid; ISmy, photoreceptor inner segment myoid; ONL, outer nuclear layer. Scale bars: 5 μ m (A, B); 20 μ m (C); 250 nm (insets).

attached, and the cone is wider than the cones in Figure 4A. The cone contains reniform mitochondria that are sparse, and a cluster of mitochondria has translocated near the nucleus (Fig. 4B, inset). In addition, cones lacking OS can have a layer of mitochondria above a devoid IS apex (Fig. 4D). This may contribute to Shed IS (discussed below). Cones with shortened IS and lacking OS contain both spheroid and reniform mitochondria (Fig. 4E). Degenerating cones found over a

FIGURE 3. Cone degeneration and mitochondria in ORT of neovascular AMD eyes. *Green arrows* indicate ELM, and *red arrowheads* indicate mitochondria in all panels. (A) Outer retinal tubulation cone with long IS containing spherical mitochondria that have not translocated internally to the ELM (*inset*, magnified box). At 2.1 mm temporal from the foveal center. Seventy-seven-year-old male donor. (B) Cone from mature⁷ ORT with nucleus retracted from ELM. Mitochondria in IS are ovoid (*inset*, magnified box). At 2 mm nasal from the foveal center. Eighty-seven-year-old female donor. (C) Cones at the mature stage of degeneration in a closed ORT⁷ Mitochondria are small and spherical (*inset*, magnified box). At 2.3 mm nasal from the foveal center. Seventy-nine-year-old male donor. (D) Cones on the scleral side of the degenerate phase, closed ORT containing translocated

mitochondria (*inset*, magnified box), and cone lipofuscin (*yellow arrowhead*). Shed IS in the lumen above the cones (*blue arrowhead*). At 2 mm nasal from the foveal center. Eighty-seven-year-old female donor. (E) High-resolution histology section from a mature⁷ ORT. External limiting membrane encircles the closed ORT. Cones have IS. Retinal pigment epithelium and other contents are seen in lumen. Cones in (C) are from this ORT. BrM, Bruch's membrane. IS, photoreceptor inner segment; ONL, outer nuclear layer. *Scale bars*: 5 μ m (A–D); 20 μ m (E); 1 μ m (*inset*).

hemorrhage in the subretinal space also contain translocated mitochondria, although sparse (Fig. 4C).

Measurements

Cone ultrastructure measurements include Mito distance, cone IS length and width in degenerating non-ORT and ORT cones, and cone ISmy in Control cones (Fig. 5). Compared to ORT cone IS length ($7.1 \pm 5.4 \mu$ m), non-ORT cone IS length ($13.3 \pm 8.3 \mu$ m), and Control cone IS length ($24.8 \pm 4.1 \mu$ m) are both longer. In ORT cones, mitochondria are closer to the ELM ($1.7 \pm 3.0 \mu$ m) than in Control cones ($12.9 \pm 4.9 \mu$ m) or non-ORT cones ($7.1 \pm 3.1 \mu$ m). Degenerating non-ORT cones were wider at the ELM ($6.3 \pm 3.2 \mu$ m) than Control cones ($4.9 \pm 1.1 \mu$ m). All three cone types differed significantly for Mito distance and cone IS length. However, the ORT cone width ($5.7 \pm 2.1 \mu$ m) was not significantly different from Control or non-ORT cone width. Cone ISmy length in Control cones was $5.2 \pm 1.7 \mu$ m. Cone ISmy was measured in unaffected cones from unremarkable eyes only, because the redistribution of mitochondria within degenerating cones rendered the ISmy undetectable.

Shed IS

In the course of this systematic review of well-preserved late AMD eyes, many with attached retinas, material with IS-like properties was found within the subretinal space and interspersed with sloughed, rounded RPE (Fig. 6A). Shed IS were found in 8 (44.4%) of the 18 neovascular AMD eyes imaged by TEM. Shed IS may be a process in cone degeneration. By viewing serial cross-section images in Figure 7, a cone can be seen shedding part of its IS into the subretinal space. Shed IS can contain small and spheroid mitochondria (Figs. 6B–D, insets). The mitochondria in Shed IS can form a cluster (Figs. 6B, 6C) or completely fill the IS material (Fig. 6D). Other Shed IS have a finely textured cytosol lacking visible organelles (Fig. 3D, blue arrowhead). We also found a cone IS apex devoid of mitochondria, with a cushion of mitochondria closer to the ELM (Fig. 4D) in an eye that also had Shed IS. This configuration may be a precursor to Shed IS.

Shed IS are distinguishable from SDD, also in the subretinal space, by its ultrastructure (Fig. 8). SDD have been described as drusenoid dollops with a granular internal structure containing unesterified cholesterol.^{36,37} Photoreceptors over Shed IS are lacking OS, whereas photoreceptors overlying SDD are more likely to have OS, although shortened.

DISCUSSION

Reflectivity of Degenerating Cone Photoreceptors

Our results provide a basis for improved interpretation of high-resolution retinal imaging including SD-OCT and AO-assisted imaging modalities such as AO-OCT^{38–43} and AOSLO.^{44–47} As seen by SD-OCT, a timeline of photoreceptor degeneration based on the disappearance of hyperreflective outer retinal bands⁴⁸ is consistent with our ultrastructural observations of cone degeneration, where cones lose OS, followed by IS shortening and mitochondrial translocation. The proposed order of photoreceptor degeneration starts with OS shortening as seen by absent band 3 (interdigitation zone), followed by band 2 (ellipsoid zone) disruption, and finally loss of band 1

(ELM).^{15,48} Although degenerating cones lose OS early like degenerating rods, ORT cones, which can survive for extended periods,⁴⁹ do so largely without OS.⁷ As seen in end-stage phase ORT, the ELM is still present when recognizable photoreceptors are absent, suggesting remarkable persistence of Müller cells and a final source of reflectivity associated with cone degeneration.⁷

A previous SD-OCT study showing decreased thickness from the outer plexiform layer to band 3 over drusen suggests a degenerative process in AMD.⁵⁰ In addition, as seen by SD-OCT, photoreceptors degenerating over small choroidal melanoma have been described as shaggy⁵¹ (i.e., irregular, swollen, and elongated). By AOSLO, reduction in cone reflectivity over drusen and at the edge of GA even when band 2 was visible on SD-OCT¹⁰ may provide insight into cone degeneration. Hyporeflexive cones at the edge of GA seen by AOSLO may show evidence of reduced waveguiding but still contain sources of IS reflectivity.¹⁰ Recently, a model developed by Zayit-Soudry et al.¹⁰ to explain observations made with AOSLO and SD-OCT in AMD showed cones thinning and retaining OS as they degenerated. Our current and published observations of short, broadened cone IS⁵ and animal models of photoreceptor degeneration do not support that interpretation.

In our separate publications, we proposed, on the basis of histology and TEM, that the reflective ORT border can be accounted for by a combination of ELM and IS mitochondria translocated to the same level as the ELM by atrophy of IS.^{7,52} In addition, we showed a direct clinicopathologic correlation between clinical SD-OCT and histology of ORT in one AMD case, establishing mitochondria as the organelle present in shrinking cone IS.⁵² These data suggest that mitochondria are independent reflectivity sources even when cones are severely misaligned, as waveguiding seems unlikely where cones are radially aligned around ORT lumens. Scattering of light by persisting mitochondria could help explain the hyperreflective ORT border seen in SD-OCT, lacking OS.⁷ Previous studies using cell culture and isolated mitochondria models suggest that mitochondria are strong light scatterers. Furthermore, light scattering by mitochondria changes when undergoing oxidative stress due to morphology and refractivity changes.^{53,54} Together with IS mitochondria, the ELM may contribute to reflectivity of degenerating non-ORT cones. The ELM of apparent ORT exhibits immunoreactivity for ICAM-1, indicating tissue injury and inflammation at this site.⁵⁵ Thickening of the ELM band is seen on SD-OCT of patients with achromatopsia, a progressive cone degeneration,⁵⁶ and Stargardt disease, caused by a dysfunctional ABCA4 protein.^{57,58} These reflectivity changes may signify mitochondria that are translocating, gliosis of Müller cells,⁵⁸ as-yet-unidentified ultrastructural alterations to junctional complexes, or a combination. Altered hyperreflectivity in SD-OCT related to mitochondria location may be good indicators of photoreceptor health. Thus, the distance between the ellipsoid zone and ELM may be an additional SD-OCT marker, which may be imaged with improved spatial resolution for cone degeneration.

Cone Degeneration

The ability to target specific cell types and organelles like we can in histology and TEM is currently not yet possible by

FIGURE 4. Mitochondria in degenerating non-ORT cones. Neovascular AMD, except where noted. *Green arrows* indicate ELM, and *red arrowheads* indicate mitochondria in all panels. (A) Non-ORT cones with long IS and OS. Mitochondria (*inset*, magnified box) are small, ovoid, and translocated in direction of the ELM leaving the IS apex devoid of any visible organelles. 0.7 mm nasal from the foveal center. Eighty-eight-year-old male donor. RPE hyperplasia of uncertain etiology. (B) Non-ORT cone with shortened IS and remnant OS (*blue arrow*). Mitochondria are sparse, and a cluster has translocated near the nucleus (*inset*, magnified box). At 3 mm from the foveal center. Seventy-seven-year-old female donor. (C) Non-ORT cone containing sparse mitochondria (*inset*, magnified box), found over a hemorrhage in subretinal space (erythrocyte, *yellow arrow*). At 1.8 mm temporal from the foveal center. Eighty-three-year-old female donor. (D) Non-ORT cone with cushion of mitochondria in IS (*inset*, magnified box). At 3 mm nasal from the foveal center. Eighty-three-year-old female donor. (E) Non-ORT cones with shortened IS containing spherical and kidney shaped mitochondria (*inset*, magnified box). At 3 mm from foveal center. Eighty-seven-year-old female donor. Scale bars: 5 μm; 1 μm (*inset*).

FIGURE 5. Cone ultrastructure measurements. Mitochondria distance (Mito distance), cone IS length, cone width, and cone IS myoid are means $\pm$ standard deviations (error bars). Cone IS myoid was measured in Control cones only because the dispersion of mitochondria within degenerating cones made myoids indistinguishable. *n*, number of cones. ANOVA. * $P < 0.001$; ** $P = 0.24$.

clinical imaging. We observed that during cone degeneration, cones lose their OS, followed by IS shortening, which have also been observed in other histological studies (summarized in Supplementary Table S1). What our study newly reveals is that

mitochondria migrate in the IS and outer fiber toward the nucleus, whereas some mitochondria are sequestered in Shed IS, sloughed off into the subretinal space. ORT and non-ORT cones degenerate in a similar but not identical fashion. Degenerating non-ORT cones are wider at the ELM than the degenerating ORT cones. Outer retinal tubulation cones may be restricted by the ELM from broadening due to space or curvature constraints related to the ELM. Degenerating non-ORT cones are located where the ELM is flat and can expand in width with fewer restrictions. Cone IS shortening and loss of detectable myoid is part of cone degeneration. Stunted, wide cones, not part of an ORT, have previously been described in AMD.^{5,59}

The material with IS properties in the subretinal space that we designated Shed IS may be a process in cone degeneration. As cones degenerate, IS shorten, perhaps by sloughing parts of the IS into the subretinal space. Depending on how the mitochondria migrate in the IS, Shed IS may or may not contain mitochondria. Shed IS were not found in all neovascular AMD eyes examined here. Why some cones Shed IS and others do not remains to be elucidated. Because we used single-section histology and TEM of advanced AMD, we may have missed Shed IS in sections not examined, or macrophages or microglia may have already cleared them. Fragmented mitochondria in cytoplasmic vacuoles have been observed undergoing extrusion from apoptotic cells, or a process called mitoptosis, in cell culture models inducing perinuclear mitochondria producing increased reactive oxygen species (ROS).^{60,61} A similar process may be occurring in photoreceptors, resulting in Shed IS.

FIGURE 6. Mitochondria in Shed IS in neovascular AMD. (A) Shed IS in subretinal space interspersed with RPE sloughed from subjacent monolayer. At 3 mm nasal from the foveal center; 0.8- μ m section with toluidine blue stain. Seventy-nine-year-old male donor. (B) Shed IS between RPE cells. Some Shed IS contain mitochondria (red arrowhead, inset, magnified box). Other Shed IS have finely textured cytosol-lacking visible organelles. At 3 mm nasal from the foveal center. Eighty-three-year-old female donor. (C) Shed IS containing a cluster of mitochondria (inset, magnified box) below non-ORT cones. ELM (green arrow). At 2.5 mm temporal from the foveal center. Seventy-six-year-old male donor. (D) Shed IS filled with mitochondria (inset, magnified box) found on top of the RPE in subretinal space. At 2.1 mm temporal from the foveal center. Seventy-nine-year-old male donor. BrM, Bruch's membrane; ELM, external limiting membrane; IS, photoreceptor inner segment; ONL, outer nuclear layer; RPE, retinal pigment epithelium. Scale bars: 20 μ m (A); 5 μ m (B–D); 1 μ m (inset).

FIGURE 7. Cone IS shedding series. Three panels showing serial high-resolution histology sections of a cone shedding part of its IS. Changes in ELM and cone size are indicated by a *green arrowhead*. BM, Bruch's membrane; ELM, external limiting membrane; IS, photoreceptor inner segment. At 3.5 mm nasal from foveal center. Ninety-year-old female donor. Neovascular AMD. Scale bar: 20 μ m.

Additionally, Shed IS may be one of several reflectivity sources in the subretinal space, along with RPE, SDD, and intact photoreceptors.

Mitochondria in Degenerating Neurons

Although our data are morphological, they are relevant to understanding subcellular mechanisms of cone degeneration potentially accessible *in vivo* via high-resolution imaging. The change in IS mitochondrial morphology from long and thin in

Control cones to small, ovoid, and reniform in degenerating cones was striking and suggests major functional change in these cells. Mitochondria are essential to oxidative phosphorylation, intermediary metabolism, calcium buffering, redox signaling, and apoptosis regulation, and are increasingly seen as central to neurodegeneration.^{62–70} Changes in all of these processes including bioenergetic failure may contribute to cell death in age-related neurodegenerations.^{69,71} Reactive oxygen species are constantly generated by oxidative phosphorylation. When levels exceed the limits of what is useful for intracellular

FIGURE 8. Shed IS in subretinal space can be distinguished from subretinal drusenoid deposit (SDD). Shed IS on left (between *red arrowheads*) with overlying degenerating non-ORT cones. Subretinal drusenoid deposit on right (between *yellow arrowheads*) with overlying photoreceptors containing OS. OPL He/ONL, ectopic photoreceptor nuclei are found translocated along the Henle fibers, blurring the division between the outer plexiform layer and outer nuclear layer at the nasal end of the section. At 3.3 mm temporal from foveal center. Ninety-four-year-old female donor. Neovascular AMD. OPL He/ONL, outer plexiform layer, Henle fibers/outer nuclear layer; ELM, external limiting membrane; INL, inner nuclear layer; RPE, retinal pigment epithelium; BLamD, basal laminar deposit.

signaling, ROS can damage mitochondrial DNA due to its vulnerable structure and may lead to mitochondrial dysfunctions including mitochondrial DNA mutations and reduction in ATP production and mitochondrial membrane potential that could cause age-related pathologies and death.^{64,72,73} Although creating larger mitochondria organelles, fusion regulated by the Mfn1, Mfn2, and OPA1 proteins, the last of which is possibly expressed in photoreceptors,^{74,75} mixes mitochondrial contents and dilutes minimally damaged components among normal organelles. While creating smaller mitochondria, fission regulated by Drp1 sequesters irreparably damaged mitochondrial components for repair or degradation by mitophagy.⁶² This process involves transport toward the soma, consistent with our observation of mitochondria translocation to the cone soma as IS shorten. Chronic fission is an early event in cell death resulting from persisting oxidative stress.⁶⁴ An increase in oxidative stress is present in mitochondrial optic neuropathies.⁶⁸ Mitochondrial dysfunctions are common in the pathogenesis of neurodegenerative diseases such as Alzheimer, Parkinson, and Huntington diseases, and amyotrophic lateral sclerosis. Our observations provide a first glimpse of apparent mitochondrial dynamism in degenerating cones in human macula.

CONCLUSIONS

This hypothesis-generating study had limitations. Transmission electron microscopy and most histological analysis used single sections only. Despite short time between death and preservation compared to most studies using human tissues, mitochondria morphology was not optimal. However, animal models commonly used to study photoreceptor degeneration do not have a cone-rich fovea for color and high-acuity vision²⁷ or photoreceptors with long Henle fibers typical of human macula. Premortem clinical imaging was not available for the eyes in this study. Nonetheless, our data provide a context for our separate descriptions of cone degeneration in ORT^{7,52} and overall fill a critical gap in the knowledge base for high-resolution clinical imaging. Furthermore, because TEM is comprehensive, our data also provide a context for targeted localizations afforded by selective markers like antibodies. In addition to imaging implications, our data provide new impetus for investigating the neurodegeneration of AMD at subcellular and molecular levels. As seen by TEM, mitochondria shrink and migrate toward the cone nucleus in degenerating cones both inside and outside ORT and are likely a major source of reflectivity observed with SD-OCT. Inner segments undergo substantial remodeling as degeneration proceeds. Our ability to follow photoreceptor degeneration in longitudinal studies of patient populations is now newly improved. Thus, photoreceptors can serve as accurate reporters of the signals coming from their support system (RPE and choroid) as it fails in AMD.

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