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“Towards Early Detection of Age-Related Macular Degeneration with Tetracyclines and FLIM,”

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Abstract

Recently, we discovered microscopic spherules of hydroxyapatite (HAP) in aged human sub-retinal pigment epithelium (sub-RPE) deposits in the retinas of aged humans (PMID:25605911), and developed evidence that the spherules may act to nucleate the growth of sub-RPE deposits such as drusen; such deposits are clinical hallmarks of and are widely believed to cause age-related macular degeneration (AMD). We found that tetracycline-family antibiotics, long known to stain HAP in teeth and bones, also stained the HAP spherules, but in general the HAP-bound fluorescence excitation and emission spectra overlapped the well-known autofluorescence of the retina, making them difficult to resolve. However, we also found that certain tetracyclines exhibited substantial increases in fluorescence lifetime upon binding to HAP, and moreover these lifetimes were substantially greater than those previously observed for autofluorescence by Schweitzer, Zinkernagel, Hammer, and others in the human retina in vivo. Thus we were able to image the HAP spherules by fluorescence lifetime imaging microscopy (FLIM) in retinas of aged humans. Implications of these findings for early diagnosis of AMD are discussed.

Keywords: FLIM, AMD, age-related macular degeneration, drusen, tetracyclines, fluorescence ophthalmoscopy

Introduction

Age-related macular degeneration remains the most common cause of blindness in the elderly in the developed world, with over ten million sufferers in the U.S. A principal clinical hallmark of the disease is the development of deposits beneath the retinal pigment epithelium (RPE) and notably in the macula; perhaps the best known of these are drusen. These deposits are widely held to induce atrophy in the neural retina in part by slowing the flux of nutrients and oxygen to, and waste products from the neural
retina. The cell death and atrophy is identified as geographic atrophy (GA), and frequently leads to choroidal neovascularization (CNV), the exudative or “wet” form of the disease. At present there is no approved treatment for GA and the disease often presents with vision loss. While genetic (e.g., CFH, APOE) and environmental (age, smoking, inflammation) risk factors are known, the etiology of most cases remains cryptic (reviews).

Recently, we discovered microscopic (ca. 1 um diameter) spherules of hydroxyapatite were present in aging retinas between the Bruch’s membrane and the RPE (Thompson, Reffatto et al. 2015); as well as larger, less symmetric nodules of HAP (Imre and Christine). These forms of calcification are distinct from the well-known calcification of the elastin layer of the Bruch’s and furthermore the spherules are often found coated with proteins identified by others as being present in sub-RPE deposits, such as vitronectin, complement factor H, and amyloid beta. This led us to propose that the spherules are pathologic and may act to nucleate the growth of the deposits; to date all sub-RPE deposits we have observed at least contain spherules. The likelihood that the spherules predate the development of deposits suggested they might serve as a useful biomarker for identifying and following the course of AMD. We found that the spherules could be imaged in vitro by fluorescence microscopy using stains (Zaheer, Lenkini et al. 2001) (Kovar, Xu et al. 2011) originally developed for studying bone growth in animal models. However, administering these stains in the eye is a challenge and they are not approved for use in human eyes. We found that certain tetracyclines offered advantages for imaging the spherules in situ using a technique called fluorescence lifetime imaging microscopy (FLIM) originally developed by two of us (Lakowicz, Szmacinski et al. 1992).

Materials and Methods

Tetracycline hydrochloride [CAS 64-75-5], Chlortetracycline [57-62-5], Minocycline [10118-90-8], and Doxycycline HCl [24390-14-5] were products of the indicated manufacturers and used without further purification. Hydroxyapatite beads (Ca₃PO₄OH, Bio Rad) or tissue sections were stained with 0.1% solutions in pH 7.5 MOPS buffer for 20-30 minutes then rinsed 2X with buffer. Human retinal tissue was obtained from anonymous, deceased donors with prior informed consent. Retinas of fixed, enucleated eyes were dissected and flat mounted with sclera and neural retina removed, and the RPE partially removed. Fluorescence lifetimes of stained HAP beads suspended in mineral oil were measured on an ISS K2 multifrequency phase fluorometer with 409 nm laser diode or 442 nm HeCd laser excitation and Rose Bengal in ethanol as a standard, essentially as previously described (Thompson and Gratton 1988); fluorescence emission spectra were obtained on a Spectronics AB-2 fluorescence spectrophotometer. Fluorescence lifetime imaging microscopy (FLIM) (Lakowicz, Szmacinski et al. 1992) was performed on an ISS Alba with 442 nm excitation, essentially as previously described (McCranor, Szmacinski et al. 2014). FLIM image data (multifrequency phase and modulation data collected for individual pixels) were fitted to multicomponent discrete component models or lifetime distributions, but most conveniently displayed in the form of phasor plots (Redford and Clegg 2005) using the manufacturer’s software. For single exponential decays the lifetime τ is a simple function of the phase shift φ or modulation m with respect to the excitation at a given circular modulation frequency ω = 2πf (Gaviola 1926):

\[ \tan \phi = \omega \tau \]
\[ m/m_{\text{exc}} = (1 + \omega^2 \tau^2)^{-1/2} \]

For multi exponential decays (e.g., where \( l(t) = \Sigma (\alpha e^{-t/\tau}) \)), the lifetimes and preexponential factors \( \alpha \) are not simple functions of the lifetime, and thus one classically collects phases and modulations over a range of modulation frequencies and fits them to an assumed decay law in an iterative process akin to
that commonly used in the time domain to recover the $\alpha$’s and $\tau$’s; see Lakowicz (Lakowicz 1999) for a fuller description. In this instance we are more interested in identifying the subset of pixels having particular lifetime(s) than accurately recovering the lifetime value, and for this purpose the phasor plot introduced by Redford and Clegg is very useful. The phase and modulation of an individual pixel at some modulation frequency is plotted in polar coordinates where the length of the vector to a point from the origin is the modulation and the angle of the vector with the x-axis is $\phi$:

![Figure 1](image.png)

**Figure 1.** Phasor plot at 100 MHz modulation frequency; the black dot ($\bullet$) indicates where a pixel with a 0.8 nsec monoexponential decay would map: $m = 0.89$, $\phi = 28.7$ degrees.

In this system a pixel exhibiting a monoexponential decay would map to the semicircular arc, whereas a pixel exhibiting a multiexponential decay would fall inside it. Pixels exhibiting similar decays would be closely grouped together, and the software enables us to highlight pixels in the image whose lifetime properties fall within a narrow range.
Results:

Fluorescence Lifetimes of HAP-bound Tetracyclines: Tetracyclines have long been known to stain bones and teeth with accompanying fluorescence, and it has been established that the tetracycline nucleus binds directly to the hydroxyapatite moiety (Skinner and Nalbandian 1975) (refs); indeed, a conjugate between tetracycline and a long wave fluorescent cyanine dye has proven highly selective as a bone stain (Kovar, Xu et al. 2011). Many tetracyclines are weakly fluorescent in solution, and the apparent increase in brightness suggested that an increase in fluorescence quantum yield and lifetime might be occurring upon tetracycline binding to HAP. Thus we stained HAP with each of the four tetracyclines above and compared the apparent lifetimes with those of the compounds in solution. Minocycline exhibited only very weak fluorescence free and bound, and tetracycline exhibited negligible lifetime differences between the free and bound forms (results not shown). However, both Doxycycline and chlortetracycline exhibited not only significant increases in intensity upon binding, but in average lifetime as well. Figure 2 depicts frequency-dependent phases and modulations for chlortetracycline and Doxycycline free and solution and bound to HAP, with the best two-component fits to the data shown. Chlortetracycline (Cl-Tet) exhibited an increase in average lifetime <τ> from 0.8 to 1.7 nsec upon HAP binding, and Doxycycline an even more pronounced increase from approximately 0.5 to 3.8 nsec. The pioneering work of Schweitzer, Zinkernagel, and their colleagues has shown that the ordinary average background autofluorescence lifetime of the human retina in vivo is rather short, typically less than 1 nsec. (Schweitzer, Quick et al. 2009; Dysli, Quellec et al. 2014), and suggests that the HAP-bound tetracycline could easily be resolved from the background on the basis of the latter’s longer lifetime.

Figure 2. Frequency-dependent phase angles (open circles) and modulations (filled circles) for chlortetracycline (left panel) and doxycycline (right panel) bound to HAP (yellow) and free in solution (blue). The lines indicate the best two component fits to the data.

Fluorescence lifetime microscopy of aged human retina specimens stained with chlortetracycline: Because the shortest wavelength excitation source available for the FLIM was 473 nm, which weakly
excited the chlortetracycline and the doxycycline not at all, we only used the former to examine human specimens. Retinas were flat-mounted, stained, and imaged by frequency domain FLIM as described above. The variations in lifetime in the image field may be displayed as maps of preexponential factors ($\alpha$) and lifetimes ($\tau$) made by fitting the frequency-dependent phases and modulations to assumed decay laws for individual pixels, or as maps of average lifetimes, but for visualizing areas of different lifetime we find it convenient to highlight a subset of pixels based on their phases and modulations using the phasor plot described above (Figure 1). This is illustrated in Figure 3, where the upper panel shows a Cl-Tet-stained fluorescence intensity image with prominent drusen, whereas in the middle panel a subset of pixels in the larger fluorescence image of Cl-Tet-stained retina having an average lifetime of 1.7 nsec corresponding to the HAP-bound Cl-Tet are circled on the phasor plot (right), and highlighted in purple. In the lower panel, pixels having a shorter average lifetime (0.6 nsec) corresponding to the background are circled and highlighted in purple. The highlighted areas in the upper panel correspond to areas of brighter fluorescence identified as drusen, although with this (20x ) objective we do not resolve individual spherules.
Figure 3. Upper panel: fluorescence intensity (excitation 473, emission 520 ± 10 nm) image of flat-mounted retina of 94 year-old woman stained with C-Tet. Middle panels: right is phasor plot with pixels having approximately 1.7 nsec lifetime circles, left is fluorescence image with pixels falling within the red circle on the phasor plot highlighted in purple. Bottom panels: right is phasor plot with red circle enclosing pixels with ~0.6 nsec lifetime, and left highlights pixels with the 0.6 nsec lifetime in purple.

Discussion

It is apparent from Figure 3 that the drusen can be resolved from the background of the retina in these fixed specimens; the background lifetime differs (is longer) from that observed by Dysli, et al., but the lifetime difference is still large enough to be distinguished. The fluorescence emission of the stained drusen is significantly greater in these fixed specimens than the background, but the spectral overlap of the tetracyclines with the background is much greater than the longer wavelength Thermo-Fisher Osteosense and LiCor Bone Tag dyes; moreover, in these fixed specimens we find that the background spectra and lifetimes differ from those observed in vivo by Dysli, et al. However, the HAP-bound lifetimes of Cl-Tet and doxycycline differ substantially from the measured retinal background lifetimes in vivo, and thus we expect to resolve the stained spherules on the basis of lifetime. While the Osteosense and Bone Tag dyes are not yet approved for human use to our knowledge, there is decades of experience with the tetracyclines in humans, and their absorption, distribution, metabolism, and excretion (ADME) in humans is well enough understood to permit their use in children. Our ultimate goal is to use this FLIM imaging approach for early detection of spherules as a means for cueing treatment of AMD with treatment(s) now under development.

Acknowledgments

References


