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Histologic Basis of Variations in Retinal Pigment Epithelium Autofluorescence in Eyes with Geographic Atrophy

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Abstract

Purpose—Lipofuscin contained in the retinal pigment epithelium (RPE) is the main source of fundus auto-fluorescence (FAF), the target of an imaging method useful for estimating the progression of geographic atrophy (GA) in clinical trials. To establish a cellular basis for hyperfluorescent GA border zones, histologic autofluorescence (HAF) was measured at defined stages of RPE pathologic progression.

Design—Experimental study.

Participants and Controls—Ten GA donor eyes (mean age $\pm$ standard deviation, 87.1 $\pm$ 4.0 years) and 3 age-matched control eyes (mean age $\pm$ standard deviation, 84.0 $\pm$ 7.2 years) without GA.

Methods—Ten-micrometer-thick sections were divided into zones of RPE morphologic features according to an 8-point scale. Any HAF excited by 488 nm light was imaged by laser confocal microscopy. The HAF intensity summed along vertical lines perpendicular to Bruch's membrane at 0.2- μ m intervals served as a surrogate for FAF. Intensity profiles in 151 zones were normalized to grade 0 at a standard reference location in each eye. Cross-sectional area, mean, and sum autofluorescence for individual RPE cells were measured (cellular autofluorescence [CAF]).

Main Outcome Measures—Statistically significant differences in intensity and localization of HAF and CAF at defined stages of RPE morphologic progression for GA and control eyes.

Results—The RPE morphologic features were most abnormal (cell rounding, sloughing, and layering; grade 2) and HAF intensity profiles were highest and most variable immediately adjacent to atrophic areas. Peaks in HAF intensity frequently were associated with vertically superimposed cells. The HAF value that optimally separated reactive RPE was 0.66 standard deviations more than the mean for uninvolved RPE and was associated with a sensitivity of 75.8% and a specificity of 76.3%. When variable cell area was accounted for, neither mean nor sum CAF differed significantly among the RPE pathologic grades.

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Conclusions—Areas with advanced RPE alterations are most likely to exhibit clinically recognizable patterns of elevated FAF around GA, but may not predict cells about to die, because of vertically superimposed cells and cellular fragments. These data do not support a role for lipofuscin-related cell death and call into question the rationale of treatments targeting lipofuscin.

Geographic atrophy (GA) resulting from age-related macular degeneration (AMD) is a sharply delimited area of atrophic retinal pigment epithelium (RPE). Age-related macular degeneration is a metabolic, vascular, and immunologic disease of the photoreceptor support system (RPE and Bruch's membrane [BrM]), with a secondary neuroretinal degeneration complicated by neovascular and exudative changes in some individuals. Geographic atrophy, considered the end-stage of AMD's underlying disease, progresses slowly,¹ causing severe and permanent vision loss on foveal encroachment.^{2,3} The number of GA patients is rising because of the aging population and continued progression of patients with neovascular AMD, despite successful treatment of choroidal neovascularization with vascular endothelial growth factor inhibitors.⁴ There are no established preventive or therapeutic options available for GA today.

To conduct studies of therapies intended to blunt GA progression, a reliable assessment of expansion rate is essential. This rate varies widely among patients (0.4–3.0 mm²/year), with a high between-eye concordance and symmetry in bilateral GA.^{1,5,6} The expansion of GA implies that RPE cells at the critical transition between health and atrophy have a high probability of dying. Thus, understanding molecular and in vivo imaging characteristics of this region is important for validating GA enlargement as a clinical end point, because these cells may be fated for death regardless of intervention. Histopathologic characterizations of the GA transition have shown cells of irregular shape and pigmentation, sloughed and heaped cells, cells in double layers, and RPE-derived material within the neurosensory retina consistent with a characteristic RPE stress-response repertoire.^{7–11}

Fundus autofluorescence (FAF) has become a valuable noninvasive tool for imaging pathologic processes affecting the RPE. Fundus autofluorescence detected at 488 nm excitation with a confocal scanning laser ophthalmoscope (cSLO) is attributable largely to RPE lipofuscin, a mixture of nondegradable material within intracellular lysosomes,^{6,12} with a minority (<10%) contribution from melanolipofuscin.¹³ This signal, in turn, is attributed to lipofuscin's main fluorophore, A2E, a bis-retinoid of the visual cycle, which involves both photoreceptors and RPE.¹² As characterized in various retinal disorders, increased and decreased FAF may have pathogenic significance and diagnostic value.^{14,15} Geographic atrophy natural history studies have suggested that sharply outlined increased FAF at the transition zone¹⁶ is an indicator for progression,^{6,16} with different FAF patterns indicating different expansion rates. Eyes without FAF abnormalities or focal increases progress only slowly, whereas banded, diffuse, or trickling patterns can progress 3 times faster.^{4,6,17}

Increased FAF can be explained by several mechanisms, including increased concentration of lipofuscin in individual cells indicated by more or brighter granules, RPE hypertrophy resulting in taller individual cells, or superimposition of cells resulting from RPE hyperplasia.^{18,19} Both hypertrophy and hyperplasia may create a longer summation path length through biologic fluorophores. Determining which mechanisms are operative in GA would be informative both for FAF interpretation and for constraining hypotheses about RPE cell death and lipofuscin's role. Lipofuscin-derived retinoid A2E has been considered toxic on the basis of focally increased FAF,^{20,21} in part underlying a therapeutic strategy designed to reduce lipofuscin in GA patients.²²

To inform the cellular basis of hyperfluorescent border zones in GA, histologic autofluorescence (HAF) was measured at defined stages of RPE pathologic progression in

sections of GA eyes. Images of RPE HAF were captured by laser confocal microscopy, using settings close to clinical parameters of cSLO (excitation, 488 nm; emission, 500+ nm).⁶ A semiquantitative grading system was used for RPE morphologic features recently used to characterize stage-specific changes in RPE protein expression.⁸ Many hyperfluorescent areas at the GA transition could be explained by vertical superimposition of RPE cells or cell-derived material.

Methods

Eyes and Tissue Preparation

All research adhered to the tenets of the Declaration of Helsinki and received institutional review board approval. Ten donor eyes (donor age range, 73–93 years) with evidence of GA were received from the Alabama Eye Bank within 6 hours after death. Globes were fixed with 4% paraformaldehyde overnight and were stored in 1% paraformaldehyde at 4°C. All eyes were examined and photographed with epi-illumination and transillumination under a preparation microscope (SMZ-U; Nikon Instruments, Inc, Melville, NY).²³ Areas of GA were measured from photographs using digital planimetry (IP Lab, version 3.9.5.r4; BD Biosciences, Exton, PA). Rectangular blocks (5×8 mm) containing the macula were cryoprotected, embedded in sucrose or optimal cutting temperature compound, frozen in liquid nitrogen, sectioned at 10 μ m (Ultracut UCT; Leica Mikrosysteme AG, Vienna, Austria), collected on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA), and air dried overnight at room temperature.²⁴ Unstained slides were coverslipped minimally with permanent mounting media (Permount; Fisher Scientific) to facilitate viewing in a single focal plane. As described,⁸ sections at 3 levels across each macula were stained with hematoxylin and eosin to determine general morphologic features, with periodic acid–Schiff hematoxylin to determine the presence of basal laminar deposits, and with von Kossa stain to determine the presence of calcium. Sections contained abundant basal deposits, drusen, or both, and did not exhibit choroidal neovascularization, supporting a diagnosis of primary GA resulting from AMD. Although it was possible theoretically that the sample included cases of GA resulting from other conditions (e.g., central areolar choroidal dystrophy²⁵), eyes with low prevalence conditions are unlikely in a screen of random donor eyes such as this one.

Microscopy and Grading of Geographic Atrophy Zones

Three representative unstained sections per eye were selected by a coauthor (R.W.R.) uninvolved in evaluation and were examined using differential interference contrast (DIC) microscopy (Eclipse 80i; Nikon Instruments, Inc). Sections crossed the atrophy and junctional zone and included normal-appearing retina at more eccentric locations. One section per eye included the optic nerve head. Differential interference contrast images were captured with a digital camera (QImaging Retiga 4000R Fast, Qcapture version 2.8.1; QImaging, Burnaby BC, Canada). Zones of RPE alterations were graded in each section according to an 8-point scale (Table 1).⁸ Separation of cell borders and thus ascertainment of stacked or layered RPE were determined by careful focus using DIC optics. Sections then were viewed with a laser confocal microscope (DM IRBM; Leica Microsystems Heidelberg GmbH, Mannheim, Germany; Leica software version 2.61). Histologic autofluorescence was measured using an oil immersion ×40 objective and the following settings: excitation, 488 nm; emission, 500+ nm (long pass); approximately 1 mW (80% energy). Image stacks were recorded in 0.5- μ m steps at a resolution of 1024×1024. Exposure settings were the same for all sections.

Image Processing, Histologic and Cellular Autofluorescence Measurements, and Normalization

From fluorescence images of histologic sections, HAF was assessed in 2 orthogonal axes (Fig 1, available at <http://aaojournal.org>). To obtain a surrogate measure for FAF, the sum HAF was calculated for each RPE pathologic zone, as follows. A maximum projection image was calculated for each image stack, rotated to align BrM horizontally, and cropped to a standard rectangular format (IP Lab, version 3.9.3r4; Biovision, Exton, PA). Then, the sum of HAF intensity was measured along vertical lines perpendicular to BrM at 0.2- μ m intervals across all RPE zones in arbitrary fluorescence units using the vertical sum intensity tool. Graphic and numerical values were exported to a spreadsheet (Microsoft Excel; Microsoft, Redmond, WA) for analysis. The calculated measure HAF sums fluorescence in an apical-to-basal direction across the entire RPE layer, whether the layer contains single or stacked cells, and thus serves as an en face view of the RPE. To compare data across eyes, HAF intensities were normalized for each eye by dividing all values by the mean HAF of a reference zone 7 mm temporal to the optic nerve head with unaltered RPE (grade 0), values called absolute HAF. Two study eyes exhibited advanced RPE atrophy with no RPE zone graded 0. The HAF data from these 2 eyes were considered in histologic descriptions only.

To obtain a measure of autofluorescence (AF) in individual RPE cells (cellular autofluorescence [CAF]), mean and sum AF values were calculated from the tissue section images, as follows. Two investigators (MR and AW) independently selected representative cells of all sizes in all AF and DIC images (mean, 10 cells per image; Leica Advanced Fluorescence version 1.9.0; Leica Microsystems). Cells with clearly distinguished boundaries in either method were outlined for assessment of cross-sectional area, mean fluorescence intensity, and sum of fluorescence intensity. Intensities were normalized to mean values at the reference location (see above).

Statistical Analysis

The primary null hypothesis was that HAF is not associated with grade of RPE morphologic features across eyes. Normalized data from 119 evaluated areas of 8 eyes were analyzed with the *F* test and 1-way analysis of variance. Mean HAF values were compared between RPE pathologic grades of 2 (abnormal) and RPE pathologic grades of 0 to 1 (normal). A receiver operating characteristic curve was used to determine sensitivity and specificity, calculated for each value observed. The sensitivity and specificity were identified for values of HAF selected a priori, that is, 1 and 2 standard deviations (SDs) more than the mean HAF of normal eyes and the HAF value that maximized sensitivity and specificity in predicting an RPE pathologic zone of 2. Grades 2A, 2B, and 2L were pooled. Observations with RPE pathologic zones of 3 or 4 were excluded from this analysis. *P* values of 0.05 or less (2-sided) were considered significant.

The secondary null hypothesis was that neither mean nor sum intensity CAF for individual RPE cells is associated with RPE grade. Mean CAF is a surrogate for intracellular fluorophore concentration. For this analysis, each individual cell's grade was considered the same as the grade of its zone. Intensities were normalized by average intensities at the reference location. Differences between grades were evaluated with a mixed-model, repeated-measures analysis of variance.

Fixation Controls

To determine if HAF and CAF measurements are affected by chemical tissue fixation, RPE AF was compared in topographically corresponding areas in each of 3 age-matched control eyes with no grossly visible retinal or pigment changes, one half of which were cryosectioned without prior fixation and the other half of which were processed after 1

month of fixation. The unfixed halves exhibited more tissue processing artifacts because of sectioning than the paraformaldehyde-fixed halves. No significant difference was found in HAF levels between these 2 methods ($P = 0.775$).

Results

Results are reported from 10 donors (7 women and 3 men) with GA (mean age, 87.1 ± 4.0 years) and 3 control donors (2 women and 1 man; mean age, 84.0 ± 7.2 years), all white. Available eye health history for 4 GA eyes indicated a clinical presentation of AMD at 1.8 to 87.8 months before death.⁸ Gross examination revealed appearances typical of GA (Fig 2, available at <http://aaojournal.org>), including central RPE atrophy and a mottled junctional zone. Some eyes had lobulated borders formed by the convergence of several small atrophic spots at the circumference of a large central area.^{8,26}

Specimens included early- to late-stage GA, as indicated by the size of the atrophic zone. Atrophy area, including atrophy within lobulated borders, varied between 2.0 and 32.0 mm² (mean, 9.9 ± 5.9 mm²; Fig 3, available at <http://aaojournal.org>; equivalent radius, 0.81–2.65 mm; mean, 1.69 ± 0.55 mm). Histologic autofluorescence at the reference location varied widely among eyes and was uncorrelated with GA size (Fig 3, available at <http://aaojournal.org>). Interestingly, HAF values at the standard location in control eyes were higher than those in GA eyes (Fig 3, available at <http://aaojournal.org>). The lowest HAF values were found in 2 eyes with the most advanced RPE degeneration and no RPE graded 0.

The RPE in all specimens was highly autofluorescent and delivered the main signal with the present exposure settings. The BrM contributed negligibly to HAF intensity in the central atrophy zone, constituting less than 5% of RPE HAF intensity. Intact hard and soft drusen exhibited negligible signal. A weak focal RPE independent signal in the photoreceptor layer in a few locations was excluded from the analysis.

Figure 4 shows typical results for grade 0 from the outer macula of GA eyes (Fig 4D–F) and control eyes (Fig 4A–C). By DIC imaging (Fig 4F), the RPE layer appears to have uniform morphologic features and pigmentation, with melanin granules at the apical surface and in the apical microvilli. The HAF at grade 0 (Fig 4E) also is uniform and very bright, with individual lipofuscin granules barely visible. There is also dim HAF associated with outer segments and BrM. Sum HAF (Fig 4D) shows limited variation, with occasional dips attributable to gaps in the sections (Fig 4D, arrow 1). Figure 4G–I shows typical results for grade 1, which features a continuous RPE layer with moderately variable cell heights (Fig 4I) and HAF (Fig 4G, H, arrows 2 and 3).

From the outer to central macula, the RPE passed steadily upward through grades of increasingly pathologic features, with stereotypic changes in the transition from grade 2 (Fig 5) to frank atrophy (Fig 6). The junctional zone was dominated by grade 2 RPE, with a discontinuous layer and large, rounded, sloughed cells next to clumped, small cells (Fig 5, cell 1 vs. cell 2). The RPE contained unevenly distributed fluorescent granules (Fig 5B). In the same eyes, HAF varied strongly and reached peaks that were several-fold higher than HAF at lower pathologic grades (Fig 5A). Where large cells were vertically superimposed, HAF was particularly high (Fig 5C, arrow 1; Fig 5D, arrow 1 and 2). These peaks in HAF contrast sharply with HAF associated with normal or small RPE cells within a monolayer, that is, not superimposed (Fig 5A, arrows). Similar effects can be seen in grade 2B (Fig 5D–F), where the RPE are basally undermined by enlarged dysmorphic cells. In Figure 5G–I, grade 2L exhibits an extended double layer of RPE (Fig 5I, dashed line) and widely varying HAF.

Grade 3, when present, was close to the atrophy margin and exhibited complete loss of basal adherence and anterior migration into the neuroretina (Fig 6A–C, arrowhead 2). Detached RPE cells were found in all retinal layers. Scattered autofluorescent debris (arrowhead 1) was particularly notable in the Henle fiber layer. The cells of grade 3 are on average large, with AF reduced compared with grade 2 RPE (Fig 7, available at <http://aaojournal.org>). Focal peaks of HAF (Fig 6A) could be attributed to vertically superimposed fluorescent cells and material.

In the central atrophic area (grade 4–5 RPE, Fig 6D–F), only sporadic cellular debris with minimal HAF is present (Fig 6D–F, arrowheads). Small autofluorescent granules are scattered in all retinal layers (Fig 6B). Thus, HAF in the atrophic area, although the lowest intensity of all grades (Fig 6), was always nonzero. The scattered granules could be residues of autolysis or phagocytosis or both, the apparent fate of grade 3 cells.

Histologic autofluorescence quantification is summarized in Figures 7 and 8 (available at <http://aaojournal.org>). Average HAF intensity was similar in grades 0 and 1 ($P = 0.12$; Fig 7, available at <http://aaojournal.org>). Among grade 2 subgrades, HAF was highest at grade 2L and 2B, which did not differ significantly from each other (Fig 8, available at <http://aaojournal.org>). Grade 2 RPE showed not only the highest intensity values (Fig 7, available at <http://aaojournal.org>; up to 2.4-fold higher than grades 0 and 1; $P = 0.01$), but also the highest variance. Sixty-four percent of RPE grade 2 showed HAF intensity profiles 1 SD larger than grades 0 and 1 RPE. Conversely, HAF intensity in 36% of zones graded 2 was identical to that of grade 0. The sensitivity and specificity were determined for increased HAF, defined as more than 1 or more than 2 SDs more than the mean of grades 0 or 1 combined, for reactive RPE of grades 2 or 3. Ideally, a diagnostic test should have both high sensitivity (few false-negative results) and high specificity (few false-positive results). The mean HAF value at grades 0 or 1 was 98.4 (SD, ± 16.9). The value at 1 SD more than this mean, or 115.3, was associated with a sensitivity of 66.7% and a specificity of 81.6% for grade 2. The value at 2 SDs more than normal, or 132.2, was associated with a sensitivity of 57.6% and a specificity of 100.0%. The optimal HAF to identify grade 2 or 3 RPE was 109.5, or 0.66 SD more than the mean for grades 0 or 1, and was associated with a sensitivity of 75.8% and a specificity of 76.3%.

Without information about the autofluorescence of individual RPE cells, it cannot be concluded that hyperfluorescence is caused exclusively by RPE cell superimposition. This possibility was assessed by examining the cross-sectional area of cells and both mean and sum CAF (Fig 9, available at <http://aaojournal.org>). The area differed among groups, with grade 2 and 3 RPE cells the largest and most variable (Fig 9A). Differences between groups in mean and sum AF (Fig 9B, C) were nonsignificant ($P > 0.05$) when cell area was considered.

Discussion

Because FAF is being advocated for imaging RPE metabolism, a more complete understanding of the sources of FAF variability and their clinical significance is essential. This study provides the first systematic and quantitative analysis of RPE HAF in AMD eyes. The principal finding is that a variability in RPE cellular morphologic features underlies the increased FAF at the transition between normal RPE and GA. Areas with advanced RPE alterations (grades 2 or 3) are most likely to cause clinically recognizable patterns of elevated FAF around atrophic GA areas, and these grades further showed the highest variation. Importantly, HAF peaks correlate with vertically superimposed RPE cells, thus providing the main cellular mechanism for increased FAF signals. Correlation of HAF intensity profiles with RPE morphologic features in this study provides insight into common

alterations associated with GA and how they can influence clinical imaging, especially FAF, but also optical coherence tomography (OCT). These results have implications for the design and interpretation of clinical trials and for the significance of RPE lipofuscin's role in AMD pathogenesis.

Advanced RPE changes (grades 2 to 3) are of particular clinical interest because they represent the predominant grades of RPE degeneration immediately adjacent to atrophy. The atrophic area border can be smooth or lobulated, with different patterns of surrounding FAF.⁶ It was shown previously in these study eyes that RPE grades 2 and 3 were prominent at both border types, with grades 2B, 2L, and 3 always closer to atrophy than grade 2A.⁸ Grades 0 and 1 dominated in the outer macula, and grades 4 and 5 dominated in the central macula. Early investigators of GA-related FAF^{20,21} thought that high border zone FAF could serve as an indicator of areas prone to cell death and subsequent atrophy expansion. However, subsequent studies showed with spatial statistics of GA border zones chosen through unbiased sampling that areas of increased FAF do not necessarily progress to atrophy²⁷ and may even predict death negatively.²⁸ These data, also using unbiased sampling, provide a basis for locally variable progression, because FAF at this border does not necessarily indicate the status of individual cells. Many dysmorphic cells were not hyperfluorescent at all, and approximately 40% of RPE zones graded 2 or 3 demonstrated HAF profiles not different from zones of normal RPE (grades 0 and 1). This finding may explain why GA does not necessarily progress in areas with highest FAF signals.²⁹ The best combined sensitivity and specificity of increased HAF for grade 2 or 3 RPE, the cells most likely to die, is approximately 75% each. These levels are considered moderate and may not meet the reliability standards required by clinical trials of GA-targeted drugs. Although FAF remains an excellent noninvasive diagnostic tool and indicator of overall RPE health,^{6,17,30} these results suggest that increased FAF alone may not reveal all areas with advanced RPE degeneration, at least with today's technology.

Cellular AF was determined as function of RPE morphologic features defined with the 8-point grading system previously used to characterize RPE protein expression across the GA transition zone of the same eyes, thus facilitating links between RPE cell biology and clinical imaging.⁸ The grading system, derived from previous histopathologic AMD stagings,^{11,23,31,32} is based on a characteristic RPE stress response that includes sloughing and anterior migration,^{33–36} double layers,^{34,37} and entrapment of RPE-derived fragments within basal deposits.^{38,39} Grade 2 or 3 cells seem to represent a response of RPE cells in loco, although migration of cells from elsewhere or proliferation remain possibilities. Although Grade 2A cells are plausible precursors of grade 3, whose remains appear scattered within retina, grades 2B and 2L may represent alternative routes to RPE death.⁸ An open question is the fate of more epithelioid-appearing RPE within grades 2 or 3. These may be slated to die as well, according to predictions of either neighbor-killing models⁴⁰ or retraction-from-free-edge⁴¹ models of RPE cell death in GA.

Previous work on these GA eyes showed reduced and abnormally localized expression of 2 proteins of the basolateral RPE plasma membrane, CD46 (a membrane-bound regulator of complement) and MCT3 (a monocarboxylate transporter), and apically expressed ezrin, a cytoplasmic peripheral membrane protein.⁸ These changes were interpreted as bellwethers for overall disturbed polarity, an essential RPE attribute,⁴² and its functional consequences. These alterations appeared between grades 0 and 1, when cells appear morphologically normal, and, according to the current results, have HAF levels that are indistinguishable from each other. Thus, molecular deterioration already is underway before focal HAF increases attributable to stage 2 RPE cells are apparent in the fundus. Conversely, these critical histologic grades (0 and 1) are not yet distinguishable clinically by FAF measurements alone.

The histologic data are potentially relevant to improving clinical FAF. First, current clinical FAF images disclose relative local differences in FAF, but do not measure FAF quantitatively, which requires the introduction of a cSLO internal fluorescent reference.⁴³ The finding of differences in absolute HAF between control and GA eyes, consistent with differences in overall fluorophore content, underscores the usefulness of using absolute FAF in a clinical setting. Second is the possibility of synergy with spectral-domain OCT, which provides detailed images of in vivo chorioretinal GA pathologic features in the same plane as the tissue sections.^{44,45} Spectral-domain OCT shows irregularities, elevations, double layers, and associated hyperreflective foci in the subretinal space and neurosensory retina,⁴⁵ remarkably similar to the RPE grades 2 and 3 described herein. Over time, these areas lose reflectivity and fade to atrophy,⁴⁵ consistent with the present interpretation of HAF. Systematic comparison of FAF-signaled RPE molecular status with retinal cross-sections revealed by spectral-domain OCT may allow RPE morphologic features to be explored in areas of high FAF using large populations of patients. A feasible near-term goal is to standardize spectral-domain OCT evaluation of RPE irregularities using a cell-based grading system like that described herein. More precise predictions of GA progression thus may be possible with combined spectral-domain OCT and FAF at corresponding locations.

That focal hyperfluorescence can be caused solely by superimposed RPE cells, as posited,^{18,19} has implications for lipofuscin cytotoxicity as a pathogenic mechanism, and thus the rationale for therapeutic strategies. Moreover, mean CAF (representing individual RPE cells, a surrogate for intracellular fluorophore concentration) did not differ significantly among RPE pathologic grades. Although limited, these data do not support the idea that concentrations of lipofuscin-derived toxins (e.g., A2E) exceed a threshold and trigger cell death. This initially attractive notion^{20,21} that excess RPE lipofuscin predisposes to atrophy expansion in vivo has not been supported. Detailed analysis reveals considerable variability in the progression of increased FAF to atrophy in AMD and Stargardt's disease.^{27,28,46} Other authors wondered how transitional RPE in GA could acquire elevated lipofuscin levels in a relatively short time frame.¹⁹ Consistent with this view is the finding that the absolute HAF values at a reference location do not indicate generally elevated HAF levels in GA (Fig 3, available at <http://aaojournal.org>). Finally, much supportive evidence for lipofuscin toxicity derives from model systems or test conditions with uncertain physiologic relevance.⁴⁷ The present data together with these and other⁴⁸ disparate lines of reasoning call into question the rationale behind therapies for GA based on reducing lipofuscin. These include modulators of RPE65, a visual cycle enzyme,²² and the synthetic retinoid fenretinide, which reduces available retinol to RPE via binding to serum retinol binding protein.⁴⁹

Strengths of this study include the range of GA stages covered, the similarity of ex vivo HAF imaging parameters to clinical cSLO, the use of a grading system to quantify RPE degeneration and DIC imaging to reveal overall tissue architecture, and an unbiased method for choosing sections to analyze. Limitations include the small number of eyes and the fact that histologic analysis by definition represents a snapshot in time. Another limitation is that HAF intensity profiles are surrogate measures that do not capture features of natural FAF such as fluorescence blocking by melanin granules in the apical RPE and the differential light path lengths through flattened versus tall RPE. A final limitation is the absence of an HAF spectral analysis that would enable discovery of contributions to overall HAF of specific bisretinoids and melanolipofuscin.^{13,50}

In conclusion, these results contribute to a cellular basis for FAF variation in GA and are useful for interpreting clinical data. Fundus autofluorescence does not display the entire extent of GA alteration. Areas with advanced RPE alterations (grade 2 or 3) are most likely to cause clinically recognizable patterns of elevated FAF around RPE atrophy zones.

Increased FAF in the junctional GA zone is attributable to superimposition of cells and cellular fragments, rather than increased accumulation of lipofuscin in individual cells. Fundus autofluorescence reveals advanced RPE alteration, but not early RPE changes, as seen by histologic examination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Sunness JS, Margalit E, Srikumaran D, et al. The long-term natural history of geographic atrophy from age-related macular degeneration: enlargement of atrophy and implications for interventional clinical trials. *Ophthalmology*. 2007; 114:271–7. [PubMed: 17270676]
2. Klein R, Meuer SM, Knudtson MD, Klein BE. The epidemiology of progression of pure geographic atrophy: the Beaver Dam Eye Study. *Am J Ophthalmol*. 2008; 146:692–9. [PubMed: 18672224]
3. Wang JJ, Rochtchina E, Lee AJ, et al. Ten-year incidence and progression of age-related maculopathy: the Blue Mountains Eye Study. *Ophthalmology*. 2007; 114:92–8. [PubMed: 17198852]
4. Rosenfeld PJ, Shapiro H, Tuomi L, et al. MARINA and ANCHOR Study Groups. Characteristics of patients losing vision after 2 years of monthly dosing in the phase III ranibizumab clinical trials. *Ophthalmology*. 2011; 118:523–30. [PubMed: 20920825]
5. Bellmann C, Jorzik J, Spital G, et al. Symmetry of bilateral lesions in geographic atrophy in patients with age-related macular degeneration. *Arch Ophthalmol*. 2002; 120:579–84. [PubMed: 12003606]
6. Holz FG, Bindewald-Wittich A, Fleckenstein M, et al. Progression of geographic atrophy and impact of fundus autofluorescence patterns in age-related macular degeneration. *Am J Ophthalmol*. 2007; 143:463–72. [PubMed: 17239336]
7. Sarks JP, Sarks SH, Killingsworth MC. Evolution of geographic atrophy of the retinal pigment epithelium. *Eye (Lond)*. 1988; 2:552–77. [PubMed: 2476333]
8. Vogt SD, Curcio CA, Wang L, et al. Retinal pigment epithelial expression of complement regulator CD46 is altered early in the course of geographic atrophy. *Exp Eye Res*. 2011; 93:413–23. [PubMed: 21684273]
9. McLeod DS, Grebe R, Bhutto I, et al. Relationship between RPE and choriocapillaris in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2009; 50:4982–91. [PubMed: 19357355]
10. Dunaief JL, Dentshev T, Ying GS, Milam AH. The role of apoptosis in age-related macular degeneration. *Arch Ophthalmol*. 2002; 120:1435–42. [PubMed: 12427055]
11. Guidry C, Medeiros NE, Curcio CA. Phenotypic variation of retinal pigment epithelium in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2002; 43:267–73. [PubMed: 11773041]
12. Sparrow JR, Hicks D, Hamel CP. The retinal pigment epithelium in health and disease. *Curr Mol Med*. 2010; 10:802–23. [PubMed: 21091424]
13. Feeney-Burns L, Hilderbrand ES, Eldridge S. Aging human RPE: morphometric analysis of macular, equatorial, and peripheral cells. *Invest Ophthalmol Vis Sci*. 1984; 25:195–200. [PubMed: 6698741]
14. Robson AG, Michaelides M, Saihan Z, et al. Functional characteristics of patients with retinal dystrophy that manifest abnormal parafoveal annuli of high density fundus autofluorescence; a review and update. *Doc Ophthalmol*. 2008; 116:79–89. [PubMed: 17985165]

15. Spaide RF, Noble K, Morgan A, Freund KB. Vitelliform macular dystrophy. *Ophthalmology*. 2006; 113:1392–400. [PubMed: 16877078]
16. Bearellly S, Cousins SW. Fundus autofluorescence imaging in age-related macular degeneration and geographic atrophy. *Adv Exp Med Biol*. 2010; 664:395–402. [PubMed: 20238040]
17. Schmitz-Valckenberg S, Fleckenstein M, Scholl HP, Holz FG. Fundus autofluorescence and progression of age-related macular degeneration. *Surv Ophthalmol*. 2009; 54:96–117. [PubMed: 19171212]
18. Spaide, RF. Chorioretinal inflammatory disorders. In: Holz, FG.; Schmitz-Valkenberg, S.; Spaide, RF.; Bird, AC., editors. *Atlas of Fundus Autofluorescence Imaging*. Springer; Berlin: 2007. p. 207-39.
19. Sparrow JR, Yoon KD, Wu Y, Yamamoto K. Interpretations of fundus autofluorescence from studies of the bisretinoids of the retina. *Invest Ophthalmol Vis Sci*. 2010; 51:4351–7. [PubMed: 20805567]
20. Holz FG, Bellman C, Staudt S, et al. Fundus autofluorescence and development of geographic atrophy in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2001; 42:1051–6. [PubMed: 11274085]
21. Bindewald A, Schmitz-Valckenberg S, Jorzik JJ, et al. Classification of abnormal fundus autofluorescence patterns in the junctional zone of geographic atrophy in patients with age related macular degeneration. *Br J Ophthalmol*. 2005; 89:874–8. [PubMed: 15965170]
22. Kubota R, Boman NL, David R, et al. Safety and effect on rod function of ACU-4429, a novel small-molecule visual cycle modulator. *Retina*. 2012; 32:183–8. [PubMed: 21519291]
23. Curcio CA, Medeiros NE, Millican CL. The Alabama Age-Related Macular Degeneration Grading System for donor eyes. *Invest Ophthalmol Vis Sci*. 1998; 39:1085–96. [PubMed: 9620067]
24. Vogt SD, Barnum SR, Curcio CA, Read RW. Distribution of complement anaphylatoxin receptors and membrane-bound regulators in normal human retina. *Exp Eye Res*. 2006; 83:834–40. [PubMed: 16764856]
25. Boon CJ, Klevering BJ, Cremers FP, et al. Central areolar choroidal dystrophy. *Ophthalmology*. 2009; 116:771–82. [PubMed: 19243827]
26. Brar M, Kozak I, Cheng L, et al. Correlation between spectral-domain optical coherence tomography and fundus autofluorescence at the margins of geographic atrophy. *Am J Ophthalmol*. 2009; 148:439–44. [PubMed: 19541290]
27. Hwang JC, Chan JW, Chang S, Smith RT. Predictive value of fundus autofluorescence for development of geographic atrophy in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2006; 47:2655–61. [PubMed: 16723483]
28. Smith RT, Gomes NL, Barile GR, et al. Lipofuscin and autofluorescence metrics in progressive STGD. *Invest Ophthalmol Vis Sci*. 2009; 50:3907–14. [PubMed: 19387078]
29. Schmitz-Valckenberg S, Bindewald-Wittich A, Dolar-Szczasny J, et al. Fundus Autofluorescence in Age-Related Macular Degeneration Study Group. Correlation between the area of increased autofluorescence surrounding geographic atrophy and disease progression in patients with AMD. *Invest Ophthalmol Vis Sci*. 2006; 47:2648–54. [PubMed: 16723482]
30. Bearellly S, Khanifar AA, Lederer DE, et al. Use of fundus autofluorescence images to predict geographic atrophy progression. *Retina*. 2011; 31:81–6. [PubMed: 20890245]
31. Sarks SH. Ageing and degeneration in the macular region: a clinico-pathological study. *Br J Ophthalmol*. 1976; 60:324–41. [PubMed: 952802]
32. van der Schaft TL, Mooy CM, de Bruijn WC, et al. Histologic features of the early stages of age-related macular degeneration: a statistical analysis. *Ophthalmology*. 1992; 99:278–86. [PubMed: 1553220]
33. Hilton GF. Subretinal pigment migration. Effects of cryosurgical retinal reattachment. *Arch Ophthalmol*. 1974; 91:445–50. [PubMed: 4827429]
34. Lopez PF, Sippy BD, Lambert HM, et al. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. *Invest Ophthalmol Vis Sci*. 1996; 37:855–68. [PubMed: 8603870]

35. Zhao C, Yasumura D, Li X, et al. mTOR-mediated dedifferentiation of the retinal pigment epithelium initiates photoreceptor degeneration in mice. *J Clin Invest*. 2011; 121:369–83. [PubMed: 21135502]
36. Hahn P, Qian Y, Dentchev T, et al. Disruption of ceruloplasmin and hephaestin in mice causes retinal iron overload and retinal degeneration with features of age-related macular degeneration. *Proc Natl Acad Sci U S A*. 2004; 101:13850–5. [PubMed: 15365174]
37. Anderson DH, Stern WH, Fisher SK, et al. The onset of pigment epithelial proliferation after retinal detachment. *Invest Ophthalmol Vis Sci*. 1981; 21:10–6. [PubMed: 7251293]
38. Kuntz CA, Jacobson SG, Cideciyan AV, et al. Sub-retinal pigment epithelial deposits in a dominant late-onset retinal degeneration. *Invest Ophthalmol Vis Sci*. 1996; 37:1772–82. [PubMed: 8759344]
39. Capon MR, Marshall J, Krafft JI, et al. Sorsby's fundus dystrophy. A light and electron microscopic study. *Ophthalmology*. 1989; 96:1769–77. [PubMed: 2482957]
40. Kaneko H, Dridi S, Tarallo V, et al. DICER1 deficit induces Alu RNA toxicity in age-related macular degeneration. *Nature*. 2011; 471:325–30. [PubMed: 21297615]
41. Gallagher-Colombo S, Maminishkis A, Tate S, et al. Expression of MCT3 expression during wound healing of the retinal pigment epithelium. *Invest Ophthalmol Vis Sci*. 2010; 51:5343–50. [PubMed: 20505202]
42. Strauss O. The retinal pigment epithelium in visual function. *Physiol Rev*. 2005; 85:845–81. [PubMed: 15987797]
43. Delori F, Greenberg JP, Woods RL, et al. Quantitative measurements of autofluorescence with the scanning laser ophthalmoscope. *Invest Ophthalmol Vis Sci*. 2011; 52:9379–90. [PubMed: 22016060]
44. Beareilly S, Chau FY, Koreishi A, et al. Spectral domain optical coherence tomography imaging of geographic atrophy margins. *Ophthalmology*. 2009; 116:1762–9. [PubMed: 19643488]
45. Fleckenstein M, Schmitz-Valckenberg S, Martens C, et al. Fundus autofluorescence and spectral-domain optical coherence tomography characteristics in a rapidly progressing form of geographic atrophy. *Invest Ophthalmol Vis Sci*. 2011; 52:3761–6. [PubMed: 21310912]
46. Chen B, Tosha C, Gorin MB, Nusinowitz S. Analysis of auto-fluorescent retinal images and measurement of atrophic lesion growth in Stargardt disease. *Exp Eye Res*. 2010; 91:143–52. [PubMed: 20398653]
47. Mainster MA, Turner PL. Blue-blocking IOLs decrease photoreception without providing significant photoprotection. *Surv Ophthalmol*. 2010; 55:272–83. [PubMed: 19883931]
48. Jackson, GR.; Curcio, CA.; Sloan, KR.; Owsley, C. Photoreceptor degeneration in aging and age-related maculopathy. In: Penfold, PL.; Provis, JM., editors. *Macular Degeneration*. Springer; Berlin: 2005. p. 45-62.
49. Meleth AD, Wong WT, Chew EY. Treatment for atrophic macular degeneration. *Curr Opin Ophthalmol*. 2011; 22:190–3. [PubMed: 21427574]
50. Sparrow JR, Gregory-Roberts E, Yamamoto K, et al. The bisretinoids of retinal pigment epithelium. *Prog Retin Eye Res*. 2012; 31:121–35. [PubMed: 22209824]

Figure 4.

Grades of minimal retinal pigment epithelium (RPE) change and associated histologic autofluorescence (HAF) in geographic atrophy (GA) and control eyes. Grades of worsening RPE pathologic features are arranged across rows. For each zone in a cryosection across a GA macula, (**C, F, I**) differential interference contrast (DIC) images of tissue morphologic features and (**B, E, H**) laser confocal microscopic images of RPE autofluorescence were obtained. **Top row**, Normalized HAF is equivalent to theoretical high-resolution amplitude of fundus confocal scanning laser ophthalmoscopy if all fluorophores in a vertically oriented band across the RPE are excited (**A, D, G**). Bar, 20 μm . **A, B, C**, Grade 0 RPE in a control eye has uniform morphologic features, pigmentation, and autofluorescence, and HAF shows minimal variation ($101.4 \pm 28.9\%$). **D, E, F**, Grade 0 RPE in a GA eye, peripheral to the atrophy zone, resembles RPE in a control eye. 1 = dip in autofluorescence; arrow = melanin granules at apical aspect. Grade 0 RPE 7 mm temporal to the optic nerve head was used for normalizing other zones. **G, E, H**, Grade 1 RPE in a GA eye showing slightly nonuniform morphologic features and autofluorescence. 2 and 3 indicate dips and rises in autofluorescence. BrM = Bruch's membrane; ELM = external limiting membrane; ONL = outer nuclear layer; PR = photoreceptors.

Figure 5.

Grades of moderate retinal pigment epithelium (RPE) change and associated histologic autofluorescence (HAF) in eyes with geographic atrophy. Same format as Figure 4. Bar, 20 μ m. **A, B, C**, Grade 2A exhibits rounded cells sloughed into the subretinal space. 1 and 2 indicate the same cells in (**B**) and (**C**). Arrowheads show peak and trough in (**A**) HAF corresponding to (**B** and **C**) the superimposition of a sloughed cell and underlying monolayer. **D, E, F**, Grade 2B is distinguished by packets of pigmented, autofluorescent material in the basal laminar deposit (asterisk, **F**) external to the RPE. Arrowheads in (**D**) align with heaped cells (3, 4 in (**E, F**)) and basally shed packets superimposed along the light path. Basal laminar deposit also contains refractile material. **G, E, H**, Grade 2L RPE is distinguished by 2 layers of cells, separated by broken lines (**H** and **I**). HAF is high and variable, corresponding to areas of superimposed cells. The ELM is intact, but photoreceptor outer segments have degenerated. *Basal laminar deposit. BrM = Bruch's membrane; DIC = differential interference contrast; ELM = external limiting membrane; IS = inner segments.

Figure 6.

Grades of severe retinal pigment epithelium (RPE) change and associated histologic autofluorescence (HAF) in eyes with geographic atrophy. Same format as Figures 4 and 5. Bar = 20 μ m. **A, B, C**, Grade 3 RPE exhibits anterior migration of pigmented, autofluorescent material into the neurosensory retina (arrowheads 1 and 2 in **(B, C)**), internal to the ELM (unnumbered arrowheads). Signal from these remnants may superimpose with signal from RPE remaining on Bruch's membrane (BrM). **D, E, F**, Grade 4 is lack of a pigmented, autofluorescent RPE layer, with basal laminar deposits remaining (asterisk). Normalized autofluorescence overall is low and is punctuated by small peaks that align with intraretinal autofluorescent material. The outer retina has degenerated severely, with loss of photoreceptor outer segments. DIC = differential interference contrast; ELM = external limiting membrane; IS = inner segments; ONL = outer nuclear layer.

Table 1**Grades of Retinal Pigment Epithelium Morphologic Features**

0	=	Uniform pigmentation and morphologic features
1	=	Nonuniform but still epithelioid morphologic features and pigmentation
2A	=	Rounding and sloughing of individual cells from the underlying substrate (either Bruch's membrane or a layer of basal deposits); anterior migration of cells within the subretinal space
2B	=	Pigmented cellular fragments within basal laminar deposit
2L	=	Double layer of continuous RPE
3	=	Anterior migration through the external limiting membrane and into neurosensory retina
4	=	Loss of pigmented cells with persisting basal laminar deposits
5	=	Loss of both pigmented cells and basal laminar deposit

RPE = retinal pigment epithelium.

Grades 0 and 1 are considered normal aging. Differentiation between grades 4 and 5 with histologic autofluorescence is not feasible because the RPE as the main fluorescence source is not present in either. These 2 grades were consolidated for data analysis.