The Relationships of Age Changes in Retinal Pigment Epithelium and Bruch’s Membrane

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PURPOSE. To study the correlations between age, Bruch’s membrane (BM) thickness, retinal pigment epithelial (RPE) autofluorescence, and RPE residual body content.

METHODS. Eight-millimeter-diameter macular discs from 88 unpaired human eye bank eyes were obtained within 72 hours of death, fixed in 10% neutral buffered formalin, and hemisected horizontally. One portion of the macular disc was embedded in paraffin and stained with periodic acid-Schiff for the measurement of BM thickness. RPE autofluorescence measurements were performed on unstained, deparaffinized sections. A second portion of the macular disc was prepared for electron microscopy to evaluate RPE residual body content. Linear and polynomial regression techniques were used to investigate the correlations between age, BM thickness, RPE autofluorescence, and RPE residual body content.

RESULTS. Bruch’s membrane thickness increased with age according to the linear model. RPE autofluorescence and RPE residual body content also increased with age, but the correlations were best approximated by a quadratic model. The correlations between RPE autofluorescence and residual body content and between BM thickness and RPE autofluorescence were best approximated by a linear regression model. There was considerable variation in these correlations between specimens and within the same age group.

CONCLUSIONS. Although the changes in RPE and Bruch’s membrane increased with age and there was a direct correlation between changes in the two tissues, there was considerable variation within each age group and between specimens. This probably reflects the multifactorial nature of the process. (Invest Ophthalmol Vis Sci. 1999;40:443-449)

Age-related macular degeneration (ARMD) accounts for approximately 50% of legal blindness in Europe and North America. The lesions causing visual loss occur as a reaction to age-related changes at the level of Bruch’s membrane (BM). Age-related thickening of BM is thought to be caused by the accumulation of waste material recognized clinically as drusen and termed age-related maculopathy (ARM). Throughout life waste material is discharged from the retinal pigment epithelium (RPE) into BM and is believed to be cleared through the choroid. It follows that dysfunction of the retinal pigment epithelium, alteration of the biophysical properties of BM, and changes in the choroidal capillaries may all influence the disorder.

There is no universal agreement about whether ARM is an exacerbation of the normal aging process or represents an entity distinct from the norm. Some evidence implies that the former view is justified. There is good evidence of genetic predisposition and it is thought that the predisposition becomes manifest if there are appropriate environmental influences. More than one gene may be involved, although the number is probably small. In these respects ARM is similar to other complex traits. The high prevalence (35%-40%) of clinically detectable ARM in people aged more than 65 years in industrial societies implies that the predisposing genes are common in the community. Furthermore, morphologic studies show a continuum of change with age. It follows that aging in the macula is probably a process that varies quantitatively and qualitatively within the community, with visual loss occurring in those with the most severe changes.

Thus, the study of the normal aging processing in the RPE, BM, and inner choroid may lead to greater understanding of the pathogenetic mechanisms of ARMD. Thickening of BM, accumulation of lipofuscin granules in the RPE, and choroidal vascular changes have been recorded with age in human macula in eye bank eyes. Based on these studies, certain correlations between age, thickness of BM, RPE autofluorescence, and RPE residual body have been established. In most studies, investigators have examined these variables individually or, at most, in combinations of two variables per study. Although a trend of change with age has been recorded, few investigators have recorded the variability within age groups. Given the multifactorial nature of the disorder, such a variation might be expected. In this study, we attempted to examine each of these variables and their intercorrelations in grossly normal human eye bank eyes. It was hoped that the findings would also provide baseline information to determine the significance of in vivo RPE autofluorescence, which can now be measured and imaged by scanning laser ophthalmoscopy.

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MATERIALS AND METHODS

Eighty-eight unpaired human eyes were obtained from the Eye Banks of the Moorfields and Bristol Eye Hospitals (donor ages, 1-98 years) within 72 hours of the donors' deaths. The eyes were fixed in 10% neutral buffered formalin. No information regarding previous eye disease was available. Eyes with macroscopically visible macular changes were excluded from the study. The globes were hemisected by a circumferential incision through the pars plana, and the anterior segments including the lens and vitreous were removed. Macular discs centered on the fovea were obtained using an 8-mm trephine.

Bruch’s Membrane Thickness

One portion of the macular disc was dehydrated, embedded in paraffin, sectioned (5 μm), and stained with periodic acid-Schiff for the measurement of Bruch’s membrane (BM) thickness. The sections were examined at ×1850 magnification using an Axiophot Universal microscope (Carl Zeiss, Oberkochen, Germany) and image management software (Tiff Image Archive System; Brian Reece Scientific, Berkshire, UK). The thickness of BM was defined as the height of the periodic acid-Schiff-positive area between the base of the RPE and the innermost portion of the subjacent choriocapillaris. A single section per case was studied, and randomized systematic sampling was used. The first point to be measured was selected at random, and thereafter, nine further measurements were made at fixed intervals. If a sample point fell on an intercapillary pillar, the measurement was made at the nearest choroidal capillary.

A second portion of the macula was fixed in 1% paraformaldehyde-3% glutaraldehyde in sodium cacodylate buffer at pH 7.4, dehydrated in acetone and propylene oxide, and embedded in Araldite for electron microscopy. Postfixation in osmic acid was avoided to facilitate the differentiation between lipofuscin and melanin granules in the RPE. Ultrathin sections were stained with uranyl acetate and lead citrate. In 69 cases, ultrastructural preservation was adequate for electron microscopic study. Ten contiguous RPE cells with underlying BM were examined with the electron microscope (model 1010; JEOL, London, UK), and electron micrographs were printed at ×6000 magnification. Montages of the RPE-BM complex were prepared. The thickness of BM was measured by dividing the cross-sectional area of BM by its length. The area was estimated by counting the number of points on a randomly placed 5-mm grid that overlaid the BM material. This included material between the RPE basal lamina and the choriocapillaris and debris in the intercapillary pillars.

RPE Autofluorescence

Deparaffinized sections immediately serial to those used for BM thickness estimates were used for the measurement of RPE autofluorescence. Autofluorescence measurements were performed on an Axiophot Universal microscope (Carl Zeiss) equipped with a 75-W mercury vapor lamp for illumination, a 450-nm to 490-nm band-pass filter, and a 520-nm low-pass barrier filter (see Fig. 2). Areas to be studied were located under bright-field illumination to minimize selection bias, and a known length of RPE was outlined. Each measurement (range, five to nine measurements per specimen) was recorded under oil immersion with a ×100 objective. Images of RPE autofluorescence were stored on disc (Tiff Image Archive System; Brian Reece) immediately after irradiation. A mean gray value of fluorescence was measured relative to a fluorescence standard (No. 474,256; Carl Zeiss) that was assigned 100 arbitrary units (AU) and a blank control that was assigned 0 AU using an image analysis system (Optima Image Analysis; Data Cell, Maidenhead, UK). The amount of autofluorescence per unit length of RPE was calculated as follows:

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\text{Autofluorescence} = \frac{\text{Mean intensity of autofluorescence} \times \text{RPE area (um}^2\text{)}}{\text{RPE length (um)}}
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Area of Residual Bodies

On the electron micrographs pigment granules were classified into those with higher electron density, designated melanin granules, and those with lower electron density, designated residual bodies (see Fig. 3). In the case of a melanolipofuscin granule, the melanosome component would be disregarded but the lipofuscin-like material was included for the purposes of assessing residual body load. The RPE cytoplasmic area and area occupied by residual bodies were determined at ×6000 magnification using a point-counting method with a 5 × 5-mm lattice. The cytoplasmic area occupied by residual bodies (in square micrometers) per unit length (in micrometers) of the RPE was then calculated.

Statistical Analyses

A pilot study was performed to identify the optimum number of measurements per section. In both light microscopy and ultrastructural morphometry, increasing the number of observations beyond 10 had no significant effect on the mean calculated for that section. Linear and polynomial regression techniques were used to investigate the correlations among age, BM thickness, RPE autofluorescence, and RPE residual body content, using a statistical analysis package (Statistica; StatSoft, Bedford, UK).

RESULTS

Age and RPE Autofluorescence

The age distribution of eye bank donors is shown in Figure 1. Reproducibility was checked by performing repeat measure-
Effect of Age in RPE and Bruch's Membrane

Figure 2. Fluorescence micrographs showing the autofluorescence in RPE of lipofuscin from eyes of a 15-year-old donor (top), a 45-year-old donor (center), and an 85-year-old donor (bottom).

Figure 3. Electron micrograph of RPE cell with photoreceptor outer segments at the top and BM at the bottom. Note numerous membrane-bound structures of intermediate electron density. To assess residual body load, the area of these was estimated and heavily pigmented melanin granules, even when part of melanolipofuscin granules (electron-dense material within a light structure), were excluded. Scale bar, 2 μm.

Reproducibility was checked by performing repeated measurements on at least two specimens freshly sectioned but previously analyzed in conjunction with each new batch of specimens prepared for the measurement of RPE autofluorescence. The average variation between these repeated measurements was 13%. RPE autofluorescence increased with age (Fig. 2), and the correlation was best approximated by a quadratic model ($P < 0.001$ for the linear and quadratic components of the model; Fig. 4). The greatest accumulation of RPE autofluorescence was observed during the first few decades of life, and accumulation reached a plateau during the eighth to ninth decades. There was considerable variation among specimens from donors of similar age, and this variation increased with age.

Age and RPE Residual Body Content

RPE residual content increased with age (Fig. 3), and the correlation was best approximated by a quadratic model ($P < 0.001$ for the linear and quadratic components of the model; Fig. 5). At any given age, RPE residual body content varied from specimen to specimen, and this variability increased with age.

Age and BM Thickness

Reproducibility was checked by performing repeated measurements on serial sections of 10 specimens. The average variation between these repeat measurements was 5%. Measurements by light microscopy showed that BM became thicker with increas-
RPE Autofluorescence and RPE Residual Body Content

A positive correlation was found between RPE autofluorescence and residual body content that was best approximated by a linear model ($P < 0.001$). There was considerable variation between specimens (Fig. 8).

RPE Changes and BM Thickness

A positive correlation was found between BM thickness, measured by light microscopy and autofluorescence that was best described by a linear regression model ($P < 0.001$; Fig. 9). A linear correlation was also identified at the electron microscopic level between BM thickness and residual body area ($P < 0.001$). The correlation was closer for autofluorescence, and for residual bodies the $R^2$ was small, implying the correlation was not close (Fig. 10).

DISCUSSION

The accumulation of residual bodies and the increase in RPE autofluorescence in the first few decades of life have been observed by others.19-21 However, Wing et al.19 found that the acquisition of RPE autofluorescence with age followed a bimodal distribution, in contrast to the near linear accumulation recorded by us in the first 7 decades of life. They reported a sharp increase in the first 2 decades of life followed by stability for 3 decades with a further increase thereafter. The discrepancy between the findings in the two studies may be caused by differences in study design. Ultraviolet excitation was per-
The observed linear correlation between RPE autofluorescence and residual body content may have been expected, given that the residual bodies are the major source of fluorescence. The variation between specimens should not be surprising because only a small proportion of the material in residual bodies fluoresces, and this proportion may be influenced by circumstances such as diet. The linear correlation between age and BM thickness are in parallel with the findings of Ramrattan et al. of eyes without ARMD, although the correlation was not precise. The $R^2$ was smaller with electron microscopic measurements than with light microscopic measurements. There were differences between the two techniques of measurement of BM thickness, which in electron microscopy included the intercapillary pillars, although we do not think that this explains the different correlations.

The possibility that the age changes in the different tissues may occur independently cannot be denied. However, it seems likely that at least some of the aspects of age-related change seen are causally related. All evidence indicates that the material in BM is derived largely from the RPE, and yet the possibility that the thickness and biochemical nature of the deposits in BM may correlate with accumulation of residual bodies and autofluorescence in the RPE had not been tested previously. Predictably, the thickness of BM would be influenced by the rate of deposition of material into it and rate of clearance. Of the latter, little is known. If the level of RPE residual body content and autofluorescence reflects metabolic activity, so may the discharge of waste material into BM. The linear correlation between BM thickness and RPE autofluorescence probably reflects this functional correlation. Our findings suggest that the correlation was better when fluorescence was considered rather than the cross-sectional area of residual bodies. This supports the suggestion that the influence of fluorescence rather than volume occupied by the residual bodies on retinal pigment epithelial function is critical to the disease process. This notion was based on the proposal that free radicals emitted by the fluorescent material may influence the composition of lipids in the RPE that would in turn make them less amenable to enzymatic degradation.
Sampling and measurement errors and genuine case-to-case differences would contribute to the variation among specimens within the specific age groups. The variation was expected to be substantial, given the apparent multifactorial nature of normal aging changes and AMD. The number of genes influencing aging in the macula is unknown, but the phenotypic variance implies that more than one gene is probably involved and that the genes conferring risk may influence different cellular functions. Furthermore, there may also be variation of environmental influences among the population in this study that predispose to AMD, although in one study it was concluded that this variation may not be great in southeastern England.

The establishment of the in vitro correlation between RPE autofluorescence and age changes in the macula at the wavelengths used in this study provides baseline information that may be relevant to the interpretation of the significance of in vivo evaluation of RPE autofluorescence by confocal scanning ophthalmoscopy. Finally, it would be of interest to compare the kinetics of age change in a population in southern England with those of eyes in which the genetic predisposition and environmental influences may differ from that which exists in our population.

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References


