Geographic atrophy (GA) of the retinal pigment epithelium (RPE) is a term used originally by Gass\(^1\) to designate one form of late age-related macular degeneration (AMD). It is the major cause of blind registration in Western communities,\(^2,3\) although, with few exceptions, it is less common than choroidal neovascular disease.\(^4-6\) The variation of phenotype implies that age-related macular degeneration (AMD) does not follow the same course from one case to another and that phenotyping may be important before initiating a therapeutic trial.

**OBJECTIVE** To document photoreceptor and retinal pigment epithelium (RPE) cell loss and other changes at the RPE-choroid interface in donated human eyes in which visual loss was deemed to be due to GA.

**RESULTS** In most of the 37 donors examined, there was marked loss of photoreceptor cells for variable distances distal from the edge of the GA. Rod loss was greater than cone loss. An inverse relationship existed between the quantity of autofluorescent inclusions in the RPE and the thickness of sub-RPE basal laminar deposit. Integrity of the choroid varied from one eye to another and was not related strictly to photoreceptor survival. In some eyes, photoreceptor loss existed in the absence of obvious morphological changes in the Bruch membrane or RPE.

**CONCLUSIONS AND RELEVANCE** The findings support the view that photoreceptor loss occurs early in AMD in a proportion of cases and imply that photoreceptor-cell loss may contribute to the functional loss recorded in early stages of AMD at least in part. The variation of changes from one eye to another implies that patients selected for a specific prophylactic therapy for early AMD should be chosen on the basis of the characteristics of their disease.

**IMPORTANCE** Geographic atrophy (GA) is the major cause of blind registration in Western communities, although, with few exceptions, it is less common than choroidal neovascular disease. The variation of phenotype implies that age-related macular degeneration (AMD) does not follow the same course from one case to another and that phenotyping may be important before initiating a therapeutic trial.
implies that that accumulation of lipofuscin in the RPE is intrinsic to the evolution of GA at least in some cases.\textsuperscript{10,11} Two studies were published in 2008 concerning the state of the photoreceptor layer in cases with GA examined in vivo with high-quality imaging using ophthalmic coherence tomography (OCT), and both came to the same conclusion.\textsuperscript{12,13} In the area of GA, there was complete or almost complete loss of photoreceptor cells. The alternatives that RPE may be absent or thinned and depigmented could not be distinguished one from the other. Beyond the edge of the GA, 2 patterns of transition were reported. In the first, there was an abrupt transition to a normal outer nuclear thickness with integrity of the line considered to be derived from the junction of the inner and outer segments of the photoreceptor cells. In the second, the photoreceptor-cell layer was thought to be thin for some distance beyond the edge of GA. In the second case, there appeared to be substantial photoreceptor loss in the retina that would have been unremarkable clinically. In neither article was the relative frequency of the 2 patterns reported.\textsuperscript{12,13} A further study was published the following year that showed similar variation in the pattern of loss and reported that both patterns of loss may occur in the same eye.\textsuperscript{14}

Psychophysical testing in early AMD consistently shows sensitivity losses in up to 25% of cases of early AMD with normal visual acuity.\textsuperscript{15-19} All studies showed that scotopic loss is greater than photopic loss, with sensitivity loss of up to 3.4 log units.\textsuperscript{15} Determining whether these losses are due to cell death or cell dysfunction was not sought.

In an attempt to establish the extent of photoreceptor-cell loss associated with GA and the topographical relation-
ship between photoreceptor and RPE cells, we examined donor eyes in which GA had been documented clinically and we related the loss to other manifestations of AMD.

Methods

The clinical records of the human eyes used in this study were obtained from MidAmerica Transplant Services (St Louis, Missouri), the Iowa Lions Eye Bank (Iowa City), and the Utah Lions Eye Bank (Salt Lake City) following written informed consent from patients. Institutional review board committee approval from the University of Utah, University of Iowa, and St Louis University for the use of human donor tissues was obtained. Most of the eyes were collected and fixed within 4 hours of death. All fundi were photographed before processing. Fundi were classified according to a modified version of the International Classification and Grading System for Age-Related Macular Degeneration. The gross pathologic features and the corresponding clinical imaging data were reviewed and graded by retinal specialists.

All donors from the repository were identified in which clinical records implied that a diagnosis of GA had been made in 1 or both eyes. The diagnosis was confirmed by examination of fundus photographs and histology, and eyes in which it was thought that visual loss may not have been due to GA were excluded from the initial survey.

Structural changes of the outer retina, BM, and choroid were documented using light, autofluorescence, and electron microscopy. For light microscopy, posterior poles were fixed in 4% paraformaldehyde in 100mM sodium cacodylate, pH 7.4, as described previously. After 2 to 4 hours of fixation, eyes were transferred to 100mM sodium cacodylate and rinsed (3 × 10 minutes), infiltrated, and embedded in acrylicm. These tissues were subsequently embedded in optimal cutting temperature medium, snap frozen in liquid nitrogen, and stored at −80°C. A few 4% paraformaldehyde–fixed eyes were embedded in paraffin and stained with hematoxylin and eosin. Tissues were sectioned to a thickness of 8 to 10 μm using a cryostat or paraffin microtome. Tissues used for transmission electron microscopic studies were fixed by immersion fixation in one-half strength Karnovsky fixative for a minimum of 24 hours. Trephine-punched specimens of the posterior pole were fixed, transferred to 100mM sodium cacodylate buffer, pH 7.4, and subsequently dehydrated, embedded in epoxy resin, sectioned, and photographed, as described previously. Tissue for confocal microscopy was prepared, as previously published.

Between 6 and 12 sections of each donor eye were examined by light and autofluorescence imaging. Special attention was paid to photoreceptor numbers, the presence of inner and outer segments, state of the RPE, BM thickness, and loss of choriocapillaris, particularly in regions distal and proximal to the edge of atrophic regions. The area (square micrometer) of basal laminar deposit (BLD) per micron of RPE/BM and the area (square micrometer) of RPE lipofuscin granules per micron of RPE basal lamina were quantified from electron micrographs. Measurements were made at 2 defined locations—1 to 2 mm and 12 to 13 mm from the foveal center—in the inferotemporal quadrant. Oriented, 4-mm diameter, full-thickness punches of RPE-choroid-sclera were taken using a trephine punch and prepared for electron microscopy, as previously described. Four random photographic images were taken from each punched specimen using a JEOL JEM 1220 microscope (JEOL USA Inc). Light microscopical images were collected at ×2500 actual magnification and electron microscopical images at ×500 actual magnification.

Results

Eyes from 80 donors were selected on the basis of clinical records. Twenty-eight were rejected after examination of fundus or gross photographs because it was judged that there was evidence of choroidal neovascularization, such as patent sub-RPE vessels, subretinal fibrosis, and/or hemorrhage, which might have preceded atrophy. In 3 donors, alternative diagnoses, such as pattern dystrophy, were considered more likely. A further 8 donors were rejected when evidence of choroidal
neovascularization within the GA was found on histopathology. In 37 donors, it was thought likely that visual loss was caused by GA and these formed the basis of this study. Of the 37 donors, 21 were female. The age range at donation was 70 to 94 years (median, 86 years). Twenty-five had bilateral GA. Of the 12 with unilateral GA, 10 had early AMD and 2 had a disciform lesion in the other eye. The remaining 4 donors lacked a clear edge of depigmentation of the fundus and it was considered that the clinical diagnosis was made on the basis of choroidal depigmentation. These eyes were also examined to test the conclusions derived from examination of the GA cases.

The edge of GA was recognized as the termination of continuous RPE. Internal to the edge there was absence of RPE cells over wide areas, although a few RPE cells were scattered over the area of GA in some specimens (Figure 1). A small number of cone nuclei were present in all cases, which were more numerous at the site of surviving RPE cells. None had inner or outer segments. In most eyes, the BM was thickened and the choriocapillaris was sparse in the region of GA, although this was quite variable from eye to eye.

In 5 donors, there was a sharp transition at the edge of the GA from a few cone nuclei to 4 to 5 rows of photoreceptor cells, both rods and cones, with inner and outer segments (Figure 2A and B). In the remaining 32 donors, there was a large transition zone in which there were only cone photoreceptors that may or may not have inner segments but no outer segments (Figures 3, 4, and 5). These were not more than 2 rows of nuclei thick. The zone extended as far as 1400 μm from the edge of the GA. Further from the edge, the outer nuclear layer became progressively thicker, cones had outer segments, and rods with outer segments appeared. The nature of the transition at the edge of the GA may vary within an eye (Figure 2). In areas lacking rods and cone outer segments, the RPE was well pigmented and contained as many autofluorescent phagolysosomes as more peripheral retina (Figures 3, 4, and 5).

The thickness of the BM varied greatly from one specimen to another (Figures 2-5). In many, the BM was only modestly thickened, whereas in others, it was markedly thickened largely owing to the presence of large quantities of BLDs. There was no obvious correlation between BM thickness and photoreceptor survival, although in none was the photoreceptor thickness normal. In those with a very thick BM, the choriocapillaris was often absent (Figure 4), whereas in the remainder, the choriocapillaris appeared to be perfused (Figure 2). In many specimens, the RPE was hyperplastic, with 2 or 3 layers of cells (Figure 5). This was most prominent in areas of reduced photoreceptor cells but was also seen where the outer nuclear layer was of normal thickness (Figure 5C).

The area of BLD per unit length of BM (a surrogate measure of BM thickness) was plotted against the area occupied by autofluorescent residual bodies over the same distance (Figure 6). There was an inverse relationship between the 2 features with a low coefficient of determination ($R^2 = 0.34$; $P = .03$), implying a weak but undoubted inverse relationship between the 2 measures. In a proportion, there was neither great thickening of BM nor excess accumulation of autofluorescence. Thus, there was variability of physical changes in choriocapillaris, BM, RPE, and photoreceptor cells relative one to another and from one eye to another. In some, there was major photoreceptor loss in the absence of morphological changes in other tissues (Figure 2E).

The eyes considered to have choroidal depigmentation all had continuous RPE. The photoreceptor layer varied from 4 to 1 nuclei in thickness, but unlike the GA cases, they contained both rods and cones (Figure 7). Also unlike GA specimens, the photoreceptor cells had outer and inner segments.
Discussion

The patterns of photoreceptor loss associated with GA in the assessed specimens are similar to those seen with OCT.12-14 We found that large numbers of photoreceptors close to the edge of GA existed in only a minority of specimens, although without serial sections of each eye it is impossible to give an accurate figure of the relative prevalence of the 2 patterns. The relative frequency of the different patterns differs from one clinical study in which an abrupt change was reported as the most common.14 The prevalence of different patterns is not stated in the other clinical studies.12,13 Differences could be explained on the basis of age. The average age of our donors was 85 years, which is greater than in the OCT studies (77.8, 72.4, and 57-80 years12-14). However, 8 of our donors were 76 years old or younger and the changes in them did not appear to be radically different from the cohort as a whole (Figure 2).

If the spatial pattern of change can be equated with the temporal course of the disease, it is evident that the demise of photoreceptor cells occurs earlier than the loss of RPE cells in most cases. This conclusion is supported by the observation that there was considerable photoreceptor loss in the eyes with choroidal depigmentation but without GA. The concept is also in accord with the conclusions drawn from previous morphological studies.7-9 It is notable that it is the rods that are more vulnerable and it appears that cones can survive for long periods without outer or inner segments. The observation of early rod loss would explain the functional loss in early AMD,15-19 implying that the functional loss in early AMD is likely to be due to photoreceptor cell death at least in part. In light of our findings, elevation of scotopic thresholds in early AMD by several thousand-fold is not surprising.

These observations are clearly important to the assessment of benefit in therapeutic trials. In patients with GA, the area of photoreceptor loss may be much greater than the area of RPE atrophy. Assuming the autofluorescence seen histopathologically is indicative of autofluorescence seen by clinical imaging, it is evident that fundus autofluorescence is not a reliable indicator of the extent or presence of photoreceptor loss. Thus, treatment trials that use visual acuity or autofluorescence delineation of RPE loss alone as a measure of therapeutic effect are not recording reliably the state of the photoreceptor cells.26-28 If the objective of treatment is directed at preservation of photoreceptors, autofluorescence imaging alone gives an incomplete record of the main therapeutic target. Other imaging techniques or functional testing would give a more complete index of therapeutic effect. This could be achieved by psychophysical measurement of rod and cone function or structural analysis using OCT, as has been

Figure 5. Autofluorescent Imaging

Photoreceptor-cell population is variable over multilayered retinal pigment epithelium. Autofluorescent imaging of a 96-year-old female donor showing areas of the retina with no (A), few (B), and normal (C) photoreceptors. In each case, the retinal pigment epithelium is more than 1 cell thick in places with considerable autofluorescence. In all, the Bruch membrane is moderately thickened and the choriocapillaris is perfused.

Figure 6. Inverse Relationship Between Bruch Membrane Thickness and Retinal Pigment Epithelium (RPE) Autofluorescence

The plot shows the cross-sectional area of the Bruch membrane plotted against the area of autofluorescent material in the RPE. There is a negative relationship between the 2 measures ($P = .03$, $R^2 = 0.34$). BLD indicates basal laminar deposit.
advocated, or adaptive optics. Perhaps the most practical approach would be assessment of bleachable rhodopsin using reflectometry. The cause of photoreceptor loss has been ascribed to changes in the RPE as originally proposed by Hogan or in the BM. The major index of aging change in the RPE is considered to be accumulation of lipofuscin, and a relationship between increased autofluorescence and risk for GA has been established. The nature of the association may be related to free radical generation by lipofuscin or failure to degrade phagosomal material and recycling lipids causing short photoreceptor outer segments. However, a large area of autofluorescent granules and a multilayered RPE were not invariably associated with photoreceptor loss in our donors (Figure 5). Concerning BM, there is considerable evidence that lipid accumulation in a thickened membrane may impede metabolic exchange between the choroid and RPE. The inverse relationship between BLD average thickness and the amount of autofluorescence might be explained on the basis of the functional relationship between the 2 entities. Reduction of metabolic exchange between the choroid and RPE would predictably cause local deficiency of vitamin A, as has been suggested in Sorsby fundus dystrophy. It has been shown that reduced availability of vitamin A in rodents reduces RPE autofluorescence.

Photoreceptor loss was also seen in eyes in which neither BM nor the RPE appeared abnormal. It is possible that functional abnormality may occur in the absence of clear structural change but the observation suggests that photoreceptor loss may be unrelated to either BM or RPE disease in some cases. One possible mechanism causing photoreceptor loss in such cases is derived from observations in the cfh knockout mouse in which there was reduced visual function in the absence of BM thickening; the BM was thinner than in aged matched mice. There was an excess of C3 protein in the outer retina and the photoreceptor outer segments were severely distorted. It was thought that there might be an imbalance between outer segment formation and outer segment shedding or of phagocytosis of shed outer segment tips. There is no clear concept of the significance of excess complement in the outer retina or the potential importance of complement to photoreceptor health. In this context, it may be relevant that the RPE expresses CFH protein inwards, CFH being a major fluid-phase regulator of the complement cascade. In addition, there is some evidence that mitochondrial changes may play a role in AMD, and it would not be surprising if such changes may affect photoreceptor cells preferentially because they have major energy requirements.

Conclusions

It is evident from our findings in eyes with GA that AMD does not follow the same course from one case to another. This should not be surprising in a multifactorial disorder in which there are multiple genetic and environmental risk factors. Proposed therapeutic approaches currently are directed toward modulating accumulation of lipofuscin in the RPE or mobilizing proteins and lipids in BM, thus causing it to become thinner. However, from structural studies, it would appear that changes in BM or the RPE are important in only a proportion of individuals with AMD. Thus, in some cases, photo-
receptor loss may be due to BM thickening, and in others, loss may be due to accumulation of lipofuscin in the RPE or loss may be unrelated to BM or RPE changes. If a specific treatment were directed to unselected cases of GA with the objective of slowing or preventing photoreceptor loss, the therapeutic approach would not address the major problem in a large proportion of cases recruited to a study, which would reduce the chance of identifying treatment benefit. As a consequence, an effective treatment of AMD may be discarded. A much greater chance of success would be achieved by robust phenotyping of cases before recruitment to a trial. Measurement of absolute levels of autofluorescence would give some measure of RPE health. It would be good if it were possible to measure the thickness of BM and its lipid content. Choroidal thickness or choroidal blood flow may give some indication as to the biophysical properties of BM. If these conclusions are correct, such measures should be used to select patients for therapeutic trials to ensure that the treatment was appropriate to the nature of the disease in those recruited. These measures would also be used to monitor therapeutic effects. Finally, in some cases, photoreceptor loss may occur in the absence of change in other tissues and may be due to disease processes intrinsic to the neuroretina. Thus, foreknowledge of the distribution and density of photoreceptor cells would be important. Clearly, it would be attractive to have a single treatment for early AMD, whereby transition to late disease were modulated, but the current evidence implies that this is unlikely to occur. It is also the case that accurate phenotyping would allow better genotype-phenotype correlation. In time, it may be shown that genotype is a good predictor of phenotype and likely disease progression. These issues will be addressed in subsequent analyses of this GA cohort.

ARTICLE INFORMATION
Submitted for Publication: January 11, 2013; final revision received June 17, 2013; accepted June 18, 2013.

Author Contributions: Drs Bird and Hageman had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Bird, Hageman.

Acquisition of data: All authors.

Analysis and interpretation of data: All authors.

Drafting of the manuscript: All authors.

Critical revision of the manuscript for important intellectual content: Bird, Hageman.

Statistical analysis: Bird, Phillips.

Obtained funding: Hageman.

Administrative, technical, and material support: Phillips, Hageman.

Study supervision: Bird, Hageman.

Conflict of Interest Disclosures: Dr Hageman is a CAB member of Seqeuorn Inc; scientific founder and shareholder for Optherion Inc; SAB member for AGTC Inc; and scientific founder and shareholder for Voyant Biotherapeutics LLC. No other disclosures were reported.

Funding/Support: This research was funded in part by National Institutes of Health grant R24-EY017404 (Dr Hageman), a grant from the American Macular Degeneration Foundation (Dr Hageman), and an unrestricted grant to the University of Utah Department of Ophthalmology and Visual Sciences (Moran Eye Center) from Research to Prevent Blindness Inc.

Role of the Sponsor: The funders had no role in the design and conduct of the study; collection, management, analysis, or interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Previous Presentation: The findings in this study formed the basis of the Charles Schepens Lecture presented at the Annual Meeting of the American Academy of Ophthalmology; October 9, 2012; Chicago, Illinois.

Additional Contributions: We are extremely grateful to Lisa Hancock, BS; Ryan Lee, DDS, Heather Stockman, BA; COT; Jill Hageman, RN; Sheri McCormick, BA; and Julie Donahue, BA, for their assistance in the processing of tissues and acquisition of donor records. Their salaries were paid, in part, by National Institutes of Health grant R24-EY017404 (Dr Hageman). We also thank the Iowa Lions and Utah Lions Eye Banks for their dedication to eye and tissue donation. This study could not have been conducted without the precious gift of eyes from donors and their families.

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