The Minnesota Grading System of Eye Bank Eyes for Age-Related Macular Degeneration

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PURPOSE. The Minnesota Grading System (MGS) is a method to evaluate human eye bank eyes and determine the level of age-related macular degeneration (AMD), by using criteria and definitions from the Age-Related Eye Disease Study (AREDS).

METHODS. Donor eyes (108 pairs) from the Minnesota Lions Eye Bank were cut circumferentially at the pars plana to remove the anterior segment. A 1000 ± 2.5-μm ruby sphere was placed on the optic nerve as a size reference. A digital, high-resolution, color macular photograph was taken through a dissecting microscope. The neurosensory retina was removed from one globe of the pair. The underlying retinal pigment epithelium was rephotographed, localizing the fovea with a proportional triangle. A grid was superimposed in the macular photographs and images were graded according to AREDS criteria. Twenty pairs were dissected bilaterally and graded for symmetry.

RESULTS. Eighty-eight globes were graded into one of four MGS categories. Nineteen (95%) of 20 globes had symmetric grades.

CONCLUSIONS. The MGS provides a methodology to grade donor tissue from eye bank eyes to correspond to the AREDS classification system. Donor tissue may be used for subsequent molecular analysis, including genomics and proteomics. (Invest Ophthalmol Vis Sci. 2004;45:4484 – 4490) DOI:10.1167/ iovs.04-0542

Age related macular degeneration (AMD) is a leading cause of visual impairment in the developed world.1-9 The lack of a comprehensive animal model of AMD limits our ability to study basic cellular mechanisms, especially in the critical early stages of age-related maculopathy (ARM) or later stages of AMD. In Minnesota, the Lions Eye Bank procures approximately 2000 globes annually. Although many eyes are used for corneal transplantation, the posterior segments from most eye bank globes are available for studying macular disease. Given the high prevalence of AMD in the general population and the age of eye bank donors (usually >55 years), eye banks represent an excellent source of tissue for studying AMD.

The Age-Related Eye Disease Study (AREDS) has described a system for classifying AMD, that is based on stereoscopic color fundus photographs and places an individual into four progressive levels, based on internationally accepted definitions.10,11 Results of the AREDS have demonstrated a protective role of antioxidant vitamins C and E, beta carotene, and zinc with copper supplementation in persons over 55 years of age with levels 3 and 4 AMD.12 The AREDS classification system10 is considered by many to be the standard for determining the level of AMD in clinical trials and is based on an extension of the Wisconsin Age-Related Maculopathy Grading System (WARMGS).13 A grading system used in the Beaver Dam Eye Study and other epidemiologic studies of AMD.14,15 The terminology of ARM and AMD can be confusing. The AREDS system describes four levels of AMD and reports level 4 (central geographic atrophy or choroidal neovascular abnormalities) as “advanced AMD.”10 The international classification considers only the “late stages of ARM” as AMD and includes either geographic atrophy or neovascularization. Finally, in the Beaver Dam Eye study separates early ARM and considers only late ARM as AMD.5

Herein, we describe the Minnesota Grading System (MGS) to classify postmortem human eye bank tissue that corresponds with the classification of AMD described in the AREDS system.10 Furthermore, by using current molecular analysis of the tissue graded with the MGS with high-throughput technologies in proteomics and genomics, along with specific immunologic analysis, valuable information of basic pathogenic mechanisms involved in each category of AMD may be obtained.

METHODS

Tissue Procurement

Donor eyes (108 pairs) were procured for the Minnesota Lions Eye Bank and placed in a moist chamber. Ages ranged from 56 to 94 years with a mean of 71 years. All tissue was acquired with consent of the donor or donor family, in accordance with the principles outlined in the Declaration of Helsinki. Investigators identified the eyes only by eye bank number, to ensure confidentiality. For most of the tissue collection during this study, eye bank procurement personnel selected globes by inquiring about a positive history of AMD with the next of kin. If an AMD history was reported, eyes were specifically transferred to our laboratory for prompt analysis. Therefore, our study group was biased to select eyes that were likely to have AMD. If the procurement personnel were able to obtain an enucleated specimen within 5 to 6 hours from time of death, the right globe was immediately (at the time of enucleation) immersed in a solution that inhibits RNA degradation (RNAlater; Ambion, Austin, TX). Due to the effect of the preservative on the globes, the eyes immersed in it were not photographed. All other globes or pairs were placed in moist chamber canisters at 4°C. Whole globes represented 62% (67/108 pairs) of our total sample.

Dissection

Globes were evaluated only if the time from death to our laboratory was 22 hours or less (mean, 10.7 hours). Eligible globes were processed immediately. Each globe was cut circumferentially at the pars plana to remove the anterior segment and provide a direct, unobstructed view of the macula. A 1000 ± 2.5-μm ruby sphere (Meller Optics, Inc., Providence, RI) was placed on the optic nerve to serve as a size reference. High-resolution, stereoscopic, color, digital fundus photographs (DXC-8500; Sony, Tokyo, Japan) were then
taken through a dissecting microscope (SMZ 1500; Nikon, Tokyo, Japan), including both the right and left macula and the optic nerve (Figs. 1A, 1B, respectively). By rotating the globe and repeating the digital image capture, we were able to produce stereoscopic images (Figs. 1C, 1D). Data generated in the present study did not rely on stereoscopic grading. Retinal tissue was carefully dissected and removed from the left globe of each pair to expose the underlying choroid and retinal pigment epithelium (RPE). The dissection technique to remove the neurosensory retina of one globe of the pair was performed without disturbing the underlying RPE and was critical to obtaining accurate photographic images of bare RPE. First, as much of the vitreous as could be safely removed was removed with Wescott scissors. Next, the neurosensory retina was grasped with 0.12-mm tissue forceps in the far periphery and gently peeled (along with the remaining vitreous) toward the optic nerve (analogous to pulling the sheets and covers off of a bed). Wescott scissors were used to excise the neurosensory retina from the optic nerve. Care was taken not to allow the tips of the scissors to touch the underlying RPE, and tissue manipulation in the macular region was avoided. If artifacts were created during dissection, they were noted and accounted for during grading. Artifacts were easy to detect, as they were usually linear and outside of the central 6 mm of the macula. “Touch artifacts” were relatively common at the edge of the optic nerve, where the neurosensory retina was excised.

Illumination

Direct, tangential, and indirect (transscleral) illumination techniques were used to highlight drusen, pigment, and vascular structures. Tangential illumination was found to highlight focal and diffuse changes in the RPE, similar to the effect of sunlight shining across a mountain range. Direct illumination of the macula was obtained by using two flexible gooseneck fiberoptic cables, each attached to a halogen light source (Schott-Fostec, Auburn, NY). The illumination was oriented tangentially, along the side of the dissecting microscope (Fig. 2). Indirect transillumination of the macula was obtained with a flatmount, plate-style, diffuse illumination system (Schott-Fostec). Specific macular features were best illustrated with a combination of illumination techniques. For example, we found that by varying the illumination, we could highlight detailed clinical features of AMD. Details of the macula were clear enough that we were able to visualize basal laminar drusen (Fig. 3).
Image Analysis

Color fundus digital images were transferred from the digital camera (with SmartMedia cards; SanDisk, Sunnyvale, CA) to a personal computer (Dual Slot Reader-Writer; Olympus, America, Inc., Lake Success, NY). Next, images were imported into an illustrator software program (Illustrator, ver. 10.0; Adobe Systems Inc., Mountain View, CA). A grid template was created digitally (Fig. 4) to match the template originally used in the early treatment of diabetic retinopathy study (ETDRS)\textsuperscript{15} and later in the AREDS.\textsuperscript{10,12} Three concentric rings were used to designate the central, inner, and outer rings. Four radiating quadrant lines were placed within the inner and outer rings to designate the superior, inferior, nasal, and temporal quadrants. The central region measures 1000 \(\mu\)m, the inner circle 3000 \(\mu\)m, and the outer ring 6000 \(\mu\)m. Using the digital imaging software program we proportionally fixed the grid overlay (i.e., changing the inner circle automatically changes the other circles proportionally, enabled through the Adobe Illustrator software) so that by matching the inner circle to the 1000-\(\mu\)m ruby sphere, all other circles would be proportionally fixed to ensure precise measurements and an accurate relationship. In addition, the grid was modified with five circles of various sizes (63, 125, 180, 360, and 660 \(\mu\)m) that were also proportionally fixed to the grid, to have an accurate size reference for grading the details of the MGS system. The template was then superimposed on the fundus image and centered on the fovea.

Symmetry

To determine the level of symmetry between eyes, 20 pairs (of the 108 pairs) or 40 eyes underwent bilateral evaluations (unmasked) both before and after retinal dissection.

Grading

Each globe was graded twice. Globes were first graded with the retina intact and then after retinal dissection, according the guidelines published for the AREDS.\textsuperscript{10} As described, we used a variety of illumination techniques, combining retro-, tangential, and direct illumination to highlight areas of drusen, depigmentation, choroidal neovascularization, fibrosis, disciform scarring, and geographic atrophy. In the AREDS, images were illuminated with shorter-wavelength light boxes. Specifically, the light boxes used “slightly bluer than northern light on a sunny day”\textsuperscript{10} to highlight features in the macula. In the MGS, we highlighted funduscopic features of AMD by using a 10% green color.
Each image was categorized as level 1, 2, 3, or 4, according to the AREDS criteria (Table 1). Globes could not be further subcategorized into grades 3a, 3b, 4a, or 4b of the AREDS system because subcategories were defined according to visual acuity criteria, which is, of course, impossible in postmortem tissue.

RESULTS

We were able to image the macula in human eye bank eyes and demonstrate most of the clinical features of AMD (Table 1). Specifically, we were able to see features consistent with small, hard drusen, basal laminar drusen, soft distinct drusen, soft indistinct drusen, calcified drusen, depigmentation, increased pigmentation, geographic atrophy, choroidal neovascularization, subretinal blood, and fibrosis. Future histopathology is needed to confirm these findings.

Some features of AMD were more difficult to visualize in eye bank eyes. Common fundus changes that occur after death include focal, subretinal, intraretinal, preretinal, or diffuse hemorrhages. Nonspecific macular hemorrhages were therefore attributed to death rather than to AMD. In addition, diffuse subretinal fluid collects in all postmortem specimens. Therefore, the presence or absence of subretinal fluid was an unreliable indicator of exudative AMD. The presence of hard exudates and subretinal fibrosis was a reliable indicator of exudative AMD.

The level of AMD was more accurately detected after dissection of the neurosensory retina (Fig. 6). We found 76 (87%) category 1, 1 (1%) category 2, 3 (3%) category 3, and 8 (9%) category 4 specimens before dissection, compared with 26 (30%) category 1, 28 (32%) category 2, 23 (26%) category 3, and 11 (12%) category 4 after dissection. In category 1, 76 globes were initially graded level 1 before dissection. After dissection, globes were recategorized mostly as level 2 (n = 28), but also as levels 3 (n = 19) and 4 (n = 3). We used the postdissection grade as the definitive assessment of the level of AMD. An example of each level of AMD is demonstrated (Fig. 7). There was a strong correlation between age and level of AMD (correlation coefficient $r = 0.67$, $P < 0.05$). There was no relationship between whole globes, posterior pole specimens, and the level of AMD (Student’s $t$-test, $P > 0.1$).

<table>
<thead>
<tr>
<th>AMD Level</th>
<th>AREDS Grading System</th>
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<tbody>
<tr>
<td>1</td>
<td>Drusen maximum size &lt; circle C-0 and total area &lt; circle C-1</td>
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<tr>
<td>2</td>
<td>(a) Drusen maximum size ≥ circle C-0 but &lt; circle C-1, or</td>
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<tr>
<td></td>
<td>(b) Drusen total area ≥ circle C-1, or</td>
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<tr>
<td></td>
<td>(c) RPE abnormalities consistent with AMD</td>
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<tr>
<td>3</td>
<td>(a) Drusen maximum size ≥ circle C-1, or</td>
</tr>
<tr>
<td></td>
<td>(b) Drusen maximum size ≥ circle C-0 and total area &gt; circle I-2 and type is soft indistinct, or</td>
</tr>
<tr>
<td></td>
<td>(c) Drusen maximum size ≥ circle C-0 and total area &gt; O-2 and type is soft distinct, or</td>
</tr>
<tr>
<td></td>
<td>(d) Geographic atrophy (atrophy &gt; circle I-1) within grid but none at center of macula</td>
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<tr>
<td>4 (advanced)</td>
<td>(a) Geographic atrophy in central subfield with at least questionable involvement of center of the macular, or</td>
</tr>
<tr>
<td></td>
<td>(b) Evidence of neovascular AMD</td>
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Size references from AREDS measurement circles: C-0 = 0.042 DD ≅ 63 μm; C-1 = 0.083 DD ≅ 125 μm; I-1 = 0.125 DD ≅ 180 μm; I-2 = 0.241 DD ≅ 360 μm; O-2 = 0.439 DD ≅ 660 μm. DD, disc diameter.

**FIGURE 5.** The proportional triangle was used to identify the fovea (•) and two prominent vascular landmarks (arrows) predissection (A) and then postdissection (B).

**TABLE 1.** AREDS Grading System
In the symmetry study, 19 (95%) globes were given the same grade. The one disparate score was grade 2 in one eye and grade 3 in the fellow eye.

**DISCUSSION**

The MGS is designed to evaluate eye bank eyes and classify the tissue according to the clinical grading system used in the AREDS.\textsuperscript{10} The MGS optimizes the clinical evaluation of eye bank tissue that may be used to study molecular events at each stage of AMD. Molecular profiling takes advantage of the dramatic technologic progress that has been made in our ability to analyze the functional gene expression and proteomic profiles of pathologic tissue. The AREDS system is used in clinical trials of AMD and is based on the Wisconsin Age-Related Maculopathy Grading System (WARMGS).\textsuperscript{13} used in the Beaver Dam Eye Study.\textsuperscript{4} The MGS provides continuity of detailed clinical definitions of AMD so that data from molecular studies will correspond to clinical and epidemiologic studies. With a mean follow-up of 6.3 years, AREDS category 1 and 2 had very low rates of progression to advanced AMD (\(<1\%\) and \(<1.3\%\) respectively). Although this is a short follow-up for an aging disorder, these early AREDS categories have an uncertain, long-term rate of progression.\textsuperscript{12}

**FIGURE 6.** Comparison of pre- and postdissection grades of AMD. This suggests that the predissection opaque retina obscures details of the drusen, thereby underestimating the level of AMD. Level 4 is either eAMD or aAMD.

**FIGURE 7.** Fundus photographs of each AMD category: (A) category 1, (B) 2, (C) 3, and (D) 4 (geographic atrophy centrally).
Curcio et al.\textsuperscript{16} have described the Alabama Grading System (AGS) for grading eye bank eyes to study the relationship of AMD with specific histopathologic findings. The AGS and the MGS have distinct objectives and should be considered complimentary, each serving a unique purpose. The AGS categorizes fixed tissue for studying the detailed histopathologic changes that occur in AMD. Although tissue-fixation techniques are essential for accurate histopathology, they also modify nucleic acids and proteins, making subsequent analysis more challenging.\textsuperscript{17} Using a general strategy for evaluating clinical tissue specimens, Gillespie et al.\textsuperscript{18} showed that the protein quantity was decreased by tissue fixation for protein analysis, compared with the use fresh frozen tissue as the standard. In our studies, the correspondence of AMD levels between paired eyes validates the symmetry in grading and validates the use of the photographed, but undissected, eye for molecular testing. The MGS categorizes donor eyes according to the AREDS. In the MGS, tissue is processed quickly and optimizes the opportunity for more accurate molecular profiling. Photographic magnification and size referencing are more accurate with the internal 1-mm ruby sphere reference. Finally, by removing opaque postmortem neurosensory retina, the details of the RPE are more easily visualized.

The MGS documents common clinical features of AMD such as small, hard drusen, intermediate drusen, soft distinct and indistinct drusen, areas of depigmentation, basal laminar drusen, hyperpigmentation, geographic atrophy, subretinal fibrosis, and choroidal neovascularization. Size and surface area are carefully controlled with the use of a standard reference (ruby sphere). Other measurements are proportionally matched to the sphere using a software program (Illustrator, ver. 10.0; Adobe Systems, Inc.). Precise foveal centration of the grid is achieved by using a proportional triangle to eliminate error that may occur due to changes in image magnification or rotation. Green filters and optimizing tissue illumination will allow investigators to identify the details of the macula required for classification of tissue according to AREDS criteria.

The MGS has limitations. Two clinical features that are not easily identified in our grading system are subretinal fluid and hemorrhage specific for AMD. Subretinal fluid accumulates in all eyes after death. Diffuse or localized subretinal, intraretinal, and preretinal hemorrhages are commonly seen in such eyes. The pattern and location of hemorrhage seen in AMD (subretinal, subfoveal, juxtafoveal, or peripapillary) helps to differentiate retinal hemorrhages related specifically to AMD. Other limitations include the time of death until globes are procured from our eye bank.\textsuperscript{20} We repli-

...to determine the grade of AMD for the pair. We elected not to dissect the fellow retina, because we found that the mechanical manipulation of the neurosensory retina may alter the protein profiles compared with the fellow undissected retina (Ferrington D, personal communication, 2004). We rely on AMD symmetry between eyes of donor pairs for levels 1 through 3. In unmasked grading, 20 globes were dissected bilaterally to determine symmetry. We found that 95% of the AMD levels were the same with only minor asymmetry in one pair (levels 2 and 3). In an evaluation of AMD in the Cardiovascular Health Study (CHS), fundus photographs of one eye were taken, and Klein et al.\textsuperscript{19} estimated that 36% of people with early and 26% of people with late AMD would be missed because of the fellow eye not being photographed. This level of asymmetry would be applicable to pairs with one eye immersed in RNA preservative (RNAlater; Ambion), because the fellow eye cannot be easily photographed. All other fellow eyes were photographable before dissection, enabling us to determine changes not detected in their uniocular analysis. Depending on retinal clarity, we are able to determine some changes in AMD in the fellow eye, particular an MGS level 4 eye before dissection.

In previous work, we have determined optimal tissue-processing conditions for studying gene expression patterns of the retina and RPE in a porcine model, designed to simulate average postmortem conditions from our eye bank.\textsuperscript{20} We replicated the standard temperature changes, tissue transportation conditions, and time intervals after procurement that occurs from donor death through final tissue analysis. We then compared the quality of gene expression analysis to fresh tissue. We determined the optimal conditions that are necessary to study the gene expression profiles of eye bank tissue.\textsuperscript{20} Specifically, we demonstrated that tissue at 5 hours after death provided good gene expression levels of "housekeeping
genes," as well as of retina- and RPE-specific genes. At 12 hours, the mRNA from the RPE was degraded, whereas the levels in the neurosensory retinal tissue remained stable up for 24 hours. The gene expression profile for RPE could be prolonged up to 24 hours by immersion of the whole globes, within 5 hours of death, directly into the preservative (RNAlater; Ambion).

In summary, we have described the Minnesota Grading System of human eye bank eyes, which is based on the AREDS grading system. We were able to identify and document detailed features of eye bank eyes by using high-resolution fundus photography and to determine the level of AMD in postmortem human tissue. We found a high degree of AMD symmetry between eyes. The MGS will identify tissue from human eyes with various levels of AMD as well as age-matched controls for molecular profiling technology. There have been recent advances in our ability to study specific protein modifications that occur in pathologic conditions.21,22 By combining sophisticated proteomic technology with a detailed system of tissue categorization (MGS), we hope to identify protein modifications unique to pathologic aging that occurs in AMD. Pathologic conditions arising from altered protein structure are referred to as "conformational disorders." These disorders have significant implications in ocular as well as other neurodegenerative conditions.53–25 Moreover, the MGS data will be relevant to ARDEs and other large clinical studies using common definitions and a standardized system. The MGS will provide a basis to improve our understanding of the pathogenesis of AMD based on understanding the molecular mechanisms involved and possibly to develop targeted therapies.

Acknowledgments

The authors thank Minnesota Lions and Minnesota Lions Eye Bank personnel, Jackie Malling, Brian Philippy, Raylene Dale, Stephanie Hackl, and the numerous tissue-procurement personnel, who assisted in acquiring the donor tissue used in this study and provide a valuable resource for the present and future studies; Jay Krachmer for expertise in designing the software toolkit used for the templates and digital imaging system and enthusiastic support of this project; and Deborah Ferrington for reviewing the manuscript.

References