The Role of Apoptosis in Age-Related Macular Degeneration

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Objective: To investigate apoptosis in human age-related macular degeneration (AMD).

Methods: Postmortem retinas with AMD and normal retinas were studied by terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) to identify dying cells, and by immunocytochemistry with cell-specific antibodies to identify rods and cones. Sections were also labeled for Fas, a cell surface receptor that triggers apoptosis in other cell types. The maculas with AMD had geographic atrophy (GA) or exudative AMD.

Results: Maculas with AMD had statistically significant increases in TUNEL-positive cells in the inner choroid, retinal pigment epithelium (RPE), photoreceptors, and inner nuclear layers compared with normal retinas. In eyes with GA, TUNEL-positive rod and RPE cell nuclei were present near edges of RPE atrophy. Photoreceptors in the maculas of eyes with AMD were strongly Fas-positive, while normal photoreceptors were only weakly labeled.

Conclusions: Evidence in this study suggests that in human AMD, RPE, photoreceptors, and inner nuclear layer cells die by apoptosis. Most TUNEL-positive RPE and photoreceptor cells were at edges of atrophy, correlating with clinically observed expansion of atrophic areas with vision loss in patients with GA. Increased Fas labeling in AMD photoreceptors indicates that the Fas/Fas ligand system may be involved in photoreceptor apoptosis. This information is essential for developing rational therapy for AMD.

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A GE-RELATED MACULAR degeneration (AMD) is the leading cause of legal blindness among people 65 years and older.1 Neovascular AMD accounts for the relatively rapid, severe vision loss that occurs in 10% of patients with AMD. The more common non-neovascular, atrophic form causes more gradual visual impairment. Patients with larger, sharply demarcated areas of atrophy have geographic atrophy (GA), which can reduce vision to the 20/200 range.2,3

Clinical and histopathologic studies have revealed that drusen, amorphous sub-retinal pigment epithelial cell (RPE) deposits, are common to all forms of AMD. The origin and composition of the drusen have been examined,4 and histopathologic analysis suggests that RPE atrophy occurs first, followed by death of overlying photoreceptors,2 with rod cell loss preceding that of cones.5,7 The mechanism of photoreceptor death is poorly understood, but one study6 found evidence of apoptosis of photoreceptors in 4 of 16 eyes with AMD using terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL).

Apoptosis, or programmed cell death, is a highly ordered and regulated cell suicide pathway that eliminates excess cells during embryonic development. Pathologic apoptosis is associated with neurodegeneration, ischemia-reperfusion injury, and bone marrow diseases.9 Typically, chromatin is fragmented and the cell shrinks as caspase enzymes degrade the cell.10 The fragmented chromatin can be detected by the TUNEL technique. Apoptosis can be divided broadly into 2 pathways. The extrinsic pathway is activated by apoptosis-inducing ligands, such as Fas ligand (FasL),11 which binds to the Fas receptor and activates a cascade of cell-digesting caspases. In the intrinsic pathway, alterations in activity and subcellular localization of Bcl-2 family members, such as Bax and Bak, alter mitochondrial membrane potential and trigger the release of cytochrome c, which activates the caspase cascade.12

Apoptosis occurs in pathologic photoreceptor cell death in several mouse models of retinal degeneration, including rd, rds, transgenic rhodopsin,13 and photic injury.14 Photoreceptor apoptosis also occurs in human retinitis pigmentosa.15

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pathologic myopia, and serous retinal detachment. Retinal ganglion cells also undergo apoptosis in glaucoma.16,17

In this study, we quantified the number of TUNEL-positive cells in each retinal layer in 10 eyes with either exudative or atrophic AMD and in 9 control eyes. To establish the identity of TUNEL-positive cells in the outer nuclear layer (ONL), we double-labeled sections with TUNEL and rod- or cone-specific antibodies. As the Fas/Fasl system has been implicated in the induction of apoptosis in several diseases, we labeled AMD sections with anti-Fas to determine if apoptosis might be triggered by the Fas/Fasl system in this disease.

**METHODS**

**SOURCE OF TISSUE AND POPULATION PROFILE**

Most postmortem eyes were obtained through the Foundation Fighting Blindness (Owings Mills, Md) eye donor program. The eye pathology reports provided the patients' age, sex, brief ocular and medical history, cause of death, and postmortem interval. Most postmortem eyes were obtained through the Foundation Fighting Blindness (Owings Mills, Md) eye donor program. Approval for research on human postmortem donor eyes was obtained from the University of Pennsylvania (Philadelphia).

**TISSUE PROCESSING AND HISTOLOGIC ANALYSIS**

On enucleation, a small incision was made in the pars plana and the eyes were fixed in a combination of 4% paraformaldehyde and 0.5% glutaraldehyde in a 0.1M phosphate buffer for several days. The fixed eyes were transferred to 2% paraformaldehyde for storage. A block of tissue containing the optic disc and macula, ranging from 0.6 to 1.5 cm in width, was processed as serial 10-µm-thick cryosections as previously described.18

**TUNEL**

TUNEL was performed on retina cryosections. Slides were dried at room temperature for 30 minutes. A hydrophobic border was created around sections with a PAP pen (Immunotech, Marseille, France) and dried for 10 minutes. Each section was rinsed with PBS twice, 2 minutes each. The positive control was incubated with 1 µg/mL of DNAse I in 50mM Tris-HCl, pH 7.5, 1mM magnesium chloride, and 1 µg/mL bovine serum albumin for 10 minutes at room temperature. The remaining samples were maintained in PBS at room temperature for 10 minutes. Sections were washed with PBS twice. TUNEL labeling was performed with an In Situ Cell Death Detection Kit, Fluorescein (Roche, Manheim, Germany). The sections were covered with paraffin, incubated for 60 minutes at 37°C, and washed with PBS 3 times for 5 minutes. Coverslips were mounted with VECTASHIELD/DAPI (Vector Labs, Burlingame, Calif).

**IMMUNOCYTOCHEMICAL ANALYSIS**

Following TUNEL labeling, sections were processed for immunocytochemical analysis as previously described.18 The pri-
Mary antibodies were antirhodopsin (4D2, 1:80; Robert Molday, PhD); a cone-specific antibody (7G6, 1:250; Peter MacLeish, PhD) and anti-Fas (CH11, 1:200; Upstate Biotechnology, Lake Placid, NY). Controls were processed with no primary antibody. Secondary antibodies were labeled with Cy-3 (1:200; Jackson ImmunoResearch Laboratories, West Grove, Pa) and examined with an Eclipse TE-300 microscope (Nikon, Japan) and a Spot RT Slider camera (Diagnostic Instruments Inc, Standish, England) with Image Pro software (Phase 3 Imaging, Glen Mills, Pa).

**Table 2. Comparison of TUNEL-Positive Cells in Retinal Layers of Age-Related Macular Degeneration Cases and Controls**

<table>
<thead>
<tr>
<th>Retinal Layers</th>
<th>No. of TUNEL-Positive Cells/5 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>IC</strong></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>6 (66.7)</td>
</tr>
<tr>
<td>Cases</td>
<td>3 (30.0)</td>
</tr>
<tr>
<td><strong>RPE</strong></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>9 (100.0)</td>
</tr>
<tr>
<td>Cases</td>
<td>1 (10.0)</td>
</tr>
<tr>
<td><strong>PR</strong></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>7 (77.8)</td>
</tr>
<tr>
<td>Cases</td>
<td>0</td>
</tr>
<tr>
<td><strong>INL</strong></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>7 (77.8)</td>
</tr>
<tr>
<td>Cases</td>
<td>1 (10.0)</td>
</tr>
<tr>
<td><strong>GCL</strong></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>7 (77.8)</td>
</tr>
<tr>
<td>Cases</td>
<td>5 (50.0)</td>
</tr>
</tbody>
</table>

*Data are given as number (percentage) of subjects. TUNEL indicates terminal deoxynucleotidyl transferase dUTP nick end-labeling; IC, inner choroid; RPE, retinal pigment epithelium; PR, photoreceptors; INL, inner nuclear layer; and GCL, ganglion cell layer.
†From the Wilcoxon exact 2-sample test.

TUNEL QUANTIFICATION

The number of TUNEL-positive cells was counted in each retinal layer in 5 sections per eye. Each section was located approximately 400 μm from the previous section and was chosen based only on its location to avoid selection bias. To be considered TUNEL-positive, each green fluorescent signal had to correspond precisely to the location of a DAPI-stained cell nucleus and be significantly brighter than the faint green background of most cell nuclei. The sum of TUNEL-positive cells in the 5 sections of each eye was adjusted for the length of the sections. For eye 00-1676, only 3 sections were available.

STATISTICAL ANALYSIS

The distribution of TUNEL-positive cells in retinal layers of AMD eyes and controls were summarized by grouped frequency distributions (Table 2). The distributions were compared using the nonparametric, Wilcoxon exact 2-sample test because of the highly skewed distribution. Data analyses were performed in SAS 8.0 (SAS Institute Inc, Cary, NC).

RESULTS

**CONTROLS FOR TUNEL STAINING OF HUMAN RETINAS**

To establish the potential of a TUNEL stain to detect apoptotic cells in human retinas, 2 controls were performed. Cryosections from several eyes with clinically normal retinas and normal postmortem histologic results (Figure 1A) were evaluated by TUNEL staining. Very weak green fluorescence was present in all nuclei (Figure 1B). Strong green autofluorescence of lipofuscin granules was apparent in the RPE cytoplasm but absent from nuclei. As a positive control, sections of normal human retinas were treated with DNAse to cleave DNA, simulating apoptotic DNA cleavage. As expected, intense green fluorescence was apparent in all nuclei (Figure 1C). On double exposure, the TUNEL signal (green) combined with the DAPI signal (blue) appears cyan.

TUNEL-POSITIVE CELLS IN AMD EYES

A survey of 9 eyes without clinical or histologic abnormalities revealed only a few TUNEL-positive cells in the inner choroid (IC), ONL, inner nuclear layer (INL), and ganglion cell layer (GCL). There were no TUNEL-positive RPE cells (Table 1). In contrast, there were statistically significant increases in TUNEL-positive cells in the AMD eyes in the IC, RPE, ONL, and INL when the data were analyzed by the Wilcoxon exact 2-sample test (Table 2). An increased number of TUNEL-positive cells was evident in 5 of 6 eyes with GA and 3 of 3 eyes with exudative AMD. The eye with drusen only (99-35) had a few TUNEL-positive cells in each nuclear layer except the GCL.

The TUNEL-positive photoreceptors in GA eyes 00-02 and 00-48 were most numerous near the edge of atrophic regions. Moving from a more normal retinal region (right side of image) to a region of atrophy (left side of image) in eye 00-02 (Figure 1D) reveals that RPE attenuation occurs first, followed by photoreceptor loss. The gross photograph of 00-02 (Figure 2B) shows extensive GA. The asterisk marks the area at the edge of atrophy corresponding to Figure 1D. The retina overlying the attenuated RPE has the greatest concentration of TUNEL-positive photoreceptors, consistent with ongoing apoptosis. TUNEL-positive photoreceptors are also evident over an area of disorganized RPE near an edge of atrophy in eye 00-48 (Figure 3A).
Figure 1. Photomicrographs showing TUNEL (terminal deoxynucleotidyl transferase dUTP nick end-labeling)-positive retinal cells in age-related macular degeneration (AMD). The bars indicate 50 µm. A-C, Controls for the TUNEL technique. Nuclei are stained with DAPI (blue). A, A normal retina labeled with mAb 7G6, with a cone-specific antibody (red). B, Lack of TUNEL labeling of a normal retina. The bright green retinal pigment epithelium (RPE) fluorescence is from lipofuscin. C, TUNEL-labeling (cyan) of all nuclei in a normal retina pretreated with DNase. D, Geographic atrophy (GA) retina 00-02 with TUNEL labeling (cyan). The nuclei are stained with DAPI (blue). The RPE is atrophic on the left. The arrows indicate a subset of TUNEL-positive photoreceptors; arrowheads, TUNEL-positive cells in the innermost inner nuclear layer; Br, Bruch membrane; and R, RPE visible due to autofluorescence of cytoplasmic lipofuscin. E, GA retina 00-02 double labeled with TUNEL (green) and mAb 7G6 (red). Autofluorescent lipofuscin in the RPE cytoplasm is at the bottom (green). The arrows indicated TUNEL-positive rods. F, GA retina 00-02 double labeled with TUNEL (green) and anti–rod opsin (red). The lipofuscin in the RPE appears gold at the bottom. The arrows indicate TUNEL-positive rods.
Most AMD eyes, such as GA eye 99-30 (Figure 3C), had fewer TUNEL-positive photoreceptors than eyes 00-02 and 00-48 described earlier but had more than the non-AMD controls. TUNEL-positive RPE cells were found most commonly near areas of atrophy and occurred more often in AMD eyes than in controls. Several TUNEL-positive RPE cells with condensed nuclei, another hallmark of apoptosis, were located near drusen (Figure 3D and E). TUNEL-positive RPE cells were also evident in eyes with exudative AMD (Figure 4D).

The INL also contained TUNEL-positive cells in both GA and exudative AMD eyes. In several AMD eyes, these cells were innermost in the INL, the zone of amacrine cells (Figures 1D and 4C). In other AMD eyes, focal pockets of TUNEL-positive cells were distributed through the full thickness of the INL (Figure 3A).

**MOST TUNEL-POSITIVE PHOTORECEPTORS ARE RODS**

To identify TUNEL-positive cells in the disorganized, degenerating ONL of AMD maculas, double staining was performed with TUNEL and rod- or cone-specific antibodies. In eye 00-02 with GA, TUNEL-positive cells near an edge of atrophy were rods; the perinuclear regions around several TUNEL-positive nuclei were marked by red fluorescence with anti-rod opsin (Figure 1F). Cones, marked by red fluorescence with mAb 7G6, a cone-specific antibody, were TUNEL-negative while a few rods in the inner part of the ONL were TUNEL-positive (Figure 1E). The TUNEL-positive photoreceptors in another GA eye (00-48) were also identified as rods by anti-rod opsin labeling (Figure 3B).

**INCREASED Fas EXPRESSION IN AMD PHOTORECEPTORS**

To determine whether expression of the Fas receptor (CD95) is altered in retinas with AMD, normal and AMD macular sections were labeled with anti-Fas, in combination with TUNEL in some eyes. The Fas antibody bound mainly to the internal limiting membrane and to the GCL in normal retinas; little labeling was found elsewhere in the retina (Figure 4A). Only a few photoreceptors were Fas-positive. In striking contrast, the AMD eyes showed Fas labeling throughout the cytoplasm of most photoreceptors (Figure 4B-D).

The increased Fas labeling in AMD photoreceptors was found in exudative AMD eyes (Figure 4B and C) and GA eyes (Figure 4D). Some photoreceptors with increased Fas labeling were also TUNEL-positive (Figure 4B).

Apoptosis is the mechanism of cell death in a number of diseases, including human retinitis pigmentosa and several mouse models of retinitis pigmentosa. An earlier study found TUNEL-positive photoreceptors in 4 of 16 eyes with AMD, suggestive of apoptosis in these cells. We have extended this finding by demonstrating a significant increase in TUNEL-positive cells in multiple retinal layers in 10 AMD eyes compared with 9 controls. The TUNEL-positive cells were located in the inner choroid, RPE, photoreceptors, and INL.

The finding of apoptosis in photoreceptors, RPE, and choroid in AMD is not surprising, as these cells are known to be lost in later stages of the disease. The clustering of TUNEL-positive photoreceptors at the edges of atrophy is consistent with a pattern of cell death and expanding GA in which RPE cells die first, followed by photoreceptor death due to loss of RPE support functions.

Most TUNEL-positive photoreceptors observed in this study were rods, as indicated by double-labeling with anti-rod opsin. This finding is consistent with the observation that rods die first in AMD. Cone rosettes over denuded RPE were observed in advanced GA, but only one cone nucleus was TUNEL-positive (data not shown).

Our finding of TUNEL-positive cells in the INL of AMD eyes was unexpected, as INL atrophy has not been reported in AMD, although one report noted some TUNEL-positive INL cells in 1 of 16 eyes tested. In our study, the TUNEL-positive cells were mainly in the inner aspect of the INL, suggesting that they may be amacrine cells. Doublelabeling with TUNEL and anti–γ amino butyric acid antiserum, designed to detect most amacrine cells, did not co-label the TUNEL-positive cells (data not shown). Perhaps the dying cells had already lost these cell-specific markers. The number of γ amino butyric acid
and glycine-positive cells in the INL is reduced in some AMD eyes (unpublished data, Ann H. Milam, PhD, 2002), consistent with attenuation of these cells in AMD. Whether such INL cell death could be related to the pathogenesis of AMD is not clear but prompts further investigation into INL changes in AMD.

Clusters of TUNEL-positive photoreceptors were present at the edges of atrophic areas in GA specimens, the area predicted to be at risk of cell death based on previous histopathologic studies. Further, the TUNEL-positive photoreceptors were rods, consistent with histopathologic studies showing that rods die before cones in AMD. However, a TUNEL signal is not always indicative of apoptosis. Cells can occasionally be TUNEL-positive owing to necrosis, a delay in tissue fixation, or antemortem hypoxia. While the mean postmortem interval for our controls (9.45 hours) was shorter than for AMD eyes (13.37 hours), the control with the longest postmortem interval (30 hours) had only 1 TUNEL-positive cell. In contrast, 1 AMD eye (00-02) with a postmortem interval of 6.5 hours had numerous TUNEL-positive cells. It seems unlikely that differences in postmortem interval alone can account for the differences in numbers of TUNEL-positive cells between AMD and control eyes. Further, the concentration of TUNEL-positive photoreceptors near edges of atrophy in GA, rather than a random distribution, suggests that the TUNEL label marks dying cells.

To further test the assertion that apoptosis occurs in AMD, we tested for changes in the levels of Fas in AMD vs control eyes. The Fas/FasL system has been implicated in the induction of apoptosis in Alzheimer disease, alcoholic hepatitis, in choroidal vascular endothelial cells following contact with RPE cells, and in RPE cells within surgically excised choroidal neovascular membranes in AMD. Photoreceptors in AMD eyes labeled more strongly with anti-Fas antibody than control eyes, where Fas was nearly undetectable in normal photoreceptors (Figure 4). The strongest Fas labeling was found in photoreceptors over fibrovascular scars or areas of RPE atrophy; these photoreceptors were most likely stressed and at risk of cell death. This finding suggests that up-regulation of Fas expression may be involved in photoreceptor apoptosis. Fas ligand expression has been detected throughout the normal mouse retina and may trigger apoptosis in photoreceptors that upregulate expression of the Fas receptor. The Fas expression in the GCL suggests that cells in this layer also may be susceptible to FasL-triggered cell death. It is also possible that the increased Fas in photoreceptors does not contribute to cell death, as some other cell types that express Fas are not susceptible to FasL-mediated cell death because they have modified downstream participants in the Fas/FasL system. However, recent evidence from Caenorhabditis elegans suggests that cell-extrinsic signals (such as FasL) can influence whether a cell with an activated apoptotic program will complete the cell death process. Further, Fas expression is increased following traumatic brain injury in humans and following exposure to RPE cells in culture to oxidative stress. Intriguingly, plasma soluble FasL is increased in patients with AMD compared with controls.

Figure 3. Photomicrographs of TUNEL (terminal deoxynucleotidyl transferase dUTP nick end-labeling)-positive cells (cyan) in eyes with age-related macular degeneration. Nuclei are stained with DAPI (blue). The bars indicate 50µm. A, Geographic atrophy (GA) eye 00-48. The arrows indicate TUNEL-positive photoreceptors and 1 TUNEL-positive cell in the choriocapillaris. The arrowhead indicates a focus of TUNEL-positive inner nuclear layer cells. B, GA eye 00-48. Rods labeled with TUNEL (green) and anti-rod opsin (red) are indicated by arrows. Autofluorescent lipofuscin in retinal pigment epithelium (RPE) cells is at the bottom. C, GA eye 99-30. Note the single TUNEL-positive photoreceptor (arrow) in an area with minimal atrophy. D, Retina with drusen but minimal atrophy (99-35). TUNEL-positive RPE nucleus (cyan, arrow) is next to a druse. Lipofuscin exhibits gold autofluorescence. E, GA eye 99-14. TUNEL-positive RPE nucleus (cyan, arrow) is overlying a soft druse.
We show that the number of TUNEL-positive cells in the choroid, RPE, ONL, and INL is significantly greater in AMD eyes vs control eyes, suggesting that these cells may die by apoptosis. The TUNEL-positive photoreceptors are clustered near areas of RPE atrophy and are mainly rods. Photoreceptors in AMD eyes upregulate Fas, a potential mediator of apoptosis, suggesting that Fas/FasL may trigger the initiation of photoreceptor apoptosis in AMD.

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