Apoptotic Photoreceptor Degeneration in Experimental Retinal Detachment

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Purpose. To investigate the possibility that cell death in retinal detachment may occur by reactivation of apoptotic programmed cell death mechanisms.

Methods. Unilateral retinal detachments were created in adult cats using 0.25% sodium hyaluronate; detached and control retinas were studied at different intervals. Internucleosomal DNA fragmentation (one of the landmarks of apoptosis) was investigated in tissue sections with the TUNEL technique, which uses terminal transferase to label with biotinylated nucleotides the 3′ ends of DNA fragments. Sections also were labeled with propidium iodide, which intensely stains pyknotic nuclei. In addition, one time point was selected for analysis with electron microscopy.

Results. TUNEL-positive (T+) and propidium iodide-positive (PI+) cells almost never were observed in retinas from control eyes, but they were abundant at defined time points after retinal detachment, appearing almost exclusively in the photoreceptor layer. Their frequency was particularly high 1 to 3 days after detachment but declined rapidly over the next several weeks. T+ cells were still present 28 days after retinal detachment. Electron microscopy also revealed evidence of apoptotic cells after retinal detachment.

Conclusions. Results are consistent with the hypothesis that photoreceptor degeneration after retinal detachment occurs through apoptosis, usually associated with intrinsic, programmed cell death mechanisms. The detection of a rapid wave of photoreceptor degeneration seems to suggest that early therapeutic interventions might be recommended; agents capable of interfering with the apoptotic mechanism could have a role in the prevention of cell losses that represent a critical complication of retinal detachment. Invest Ophthalmol Vis Sci. 1995;36:990–996.

Retinal detachment (RD) is a serious clinical problem characterized by separation of the sensory retina from the RPE with fluid accumulation in the intervening space. The incidence of RD is approximately 5 to 12 persons per 100,000 per year for phakic, nontraumatic forms[1]; traumatic RD is seen most frequently among men.[2] Primary RD is usually preceded by posterior vitreous detachment,[1] which is relatively rare in persons younger than 30 years of age, but it affects as many as 63% of people older than 70.[3]

Because of the scarcity of human tissue samples, much of the knowledge concerning histologic features and pathophysiology of RD derives from animal studies in nonhuman primates.[4] Some advantages to using animal models include more precise control of the creation and location of the RD, the extent of the RD, and the removal of the eye for analysis. In addition, it is possible to create RD in multiple animals at many different time points, which is not possible in preserved human tissue. Early alterations after RD include changes in protein synthesis[5–8] and intraretinal edema, most notably in the inner nuclear layer.[4,9] There is early disorganization of photoreceptor outer segment disks,[10,11] followed by decreases in the amount of disk material.[10,11] Some RPE cells become enlarged, separate from Bruch's membrane, and even-

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tually are seen in the subretinal fluid, vitreous cavity, and on both retinal surfaces.[9,11,12]

Cats have also been used as models for RD studies; similarities between human and cat retinas include extensive capillary networks[13] and the differential distribution of cones in specialized regions of the retina.[14] Cat retinal cell responses to RD have been studied extensively.[15–16] Early changes include the appearance of mitotic figures within the RPE cell layers and of polymorphonuclear neutrophils, monocytes, photoreceptor cell bodies and outer segments, Müller cells, and RPE cells within the subretinal space.[17] Significant decreases in the number of photoreceptor nuclei have been observed between days 13 and 30. Although rods apparently degenerate faster than cones, the latter are not spared because no receptor terminals are seen 50 days after RD.[18]

The mechanisms of photoreceptor death after RD remain obscure.[18] One possible mechanism is *necrosis*; it usually occurs after tissue injury, affects groups of cells, and results in an inflammatory response. An alternative mechanism is *apoptosis*, frequently associated with programmed cell death, which affects individual cells and does not trigger inflammatory responses. Apoptotic cells usually are phagocytosed by neighboring cells, not by migratory immune cells. The recent finding that apoptosis is the mechanism through which photoreceptors degenerate in several animal models of retinitis pigmentosa[19–22] suggested the possibility that it could also be involved in RD. We have tested this hypothesis, taking advantage of the fact that apoptosis can be distinguished readily from necrosis by the occurrence of internucleosomal DNA fragmentation, which can be visualized by the presence of a characteristic DNA ladder using agarose gel electrophoresis[23] and/or in situ labeling with terminal deoxynucleotidyl transferase (TdT)-mediated incorporation of biotionylated nucleotides into the 3′ ends of DNA fragments (terminal dUTP nick end labeling [TUNEL]).[24] Our findings suggest the involvement of an apoptotic mechanism in the degeneration of photoreceptor cells after retinal detachment.

METHODS

Creation of Experimental Retinal Detachment

All investigations using animals were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee at the University of California at Santa Barbara, where the surgery was performed.

Unilateral RD in adult cats was performed as described by Anderson et al.[25] After extracapsular lens extraction and a 2-week healing period, an Ocutome (CooperVision, Irvine, CA) was used to remove the posterior lens capsule and vitreous. A fluid–gas exchange was then performed. Using a glass pipette with a flat 80- to 100-μm tip diameter, 0.25% sodium hyaluronate (0.5 mg/ml Healon; Pharmacia, Uppsala, Sweden) was injected slowly as the pipette was advanced into the retina. When the pipette tip reached the subretinal space, a small bleb formed, creating a unilateral retinal detachment. The size of the retinal detachment was regulated by the amount of sodium hyaluronate injected. The contralateral eye remained unoperated and was used as a control. Detached and control retinas were fixed either 1, 3, 7, 14, or 28 days after surgery. After immersion fixation in 4% paraformaldehyde, retinas were embedded in paraffin and sectioned at 4 μm.

DNA Nick End Labeling by the TUNEL Method

Sections were deparaffinized by heating at 70°C for 10 minutes and washing twice in xylene for a total of 10 minutes, and they were rehydrated through a graded series of alcohols and double-distilled water (ddH_2O). The TUNEL technique was performed as described by Gavrieli et al,[24] with some minor modifications.[19] Briefly, tissue sections were treated with proteinase K (20 μg/ml) in 10 mM tris HCl (pH 8.0) for 15 minutes at room temperature and washed four times for 2 minutes in ddH_2O. Endogenous peroxidase were inactivated by incubating the sections for 5 minutes in 3% H_2O_2 at room temperature and then washing three times in ddH_2O. Sections were preincubated for 10 minutes at room temperature in TdT buffer (30 mM tris HCl [pH 7.2]–140 mM sodium cacodylate–1 mM cobalt chloride) and incubated in a moist chamber for 1 hour at 37°C with 20 to 30 μl of TdT buffer with 0.5 U TdT/1 μl and 40 μM biotinylated 16-dUTP. The reaction was stopped by transferring the sections to 2 × SSC buffer (300 mM NaCl–30 mM sodium citrate) for 15 minutes at room temperature. The sections were washed for 5 minutes in 1 × phosphate-buffered saline (PBS) and blocked in 2% bovine serum albumin in PBS for 10 minutes at room temperature. After rinsing in ddH_2O, the sections were washed in PBS for 5 minutes and incubated for 30 minutes at 37°C in Vectastain ABC peroxidase standard solution (Vector Laboratories, Burlingame, CA), rinsed twice in PBS, and stained for 30 minutes at 37°C using aminoethylcarbamazole as a substrate. After the developing reaction was stopped with water, the sections were coverslipped using Aqua-Poly/Mount (Polysciences, Warrington, PA). Positive controls were incubated with DNase I (1 μg/ml) in TdT buffer for 10 minutes at room temperature before the incubation in biotinylated nucleotides. DNase, RNase,

and biotin 16-dUTP were purchased from Boehringer Mannheim (Indianapolis, IN).

Quantitative Analysis

Three to four eyes were used for most experimental retinal detachment time points (1, 3, 7, and 28 days after retinal detachment). The exception was the 14-day time point, from which only two eyes were available for analysis. Four unoperated control eyes from four different animals also were analyzed. The number of labeled cells per section in each nuclear layer was counted in approximately 25 sections from each time point and was expressed per square millimeter of tissue; tissue areas were measured using a calibrated ocular micrometer. Results are expressed as mean ± standard deviation.

Propidium Iodide Labeling

Propidium iodide (PI) has been used in many studies as a marker for cell death.[26-28] Deparaffinized, rehydrated sections were incubated with 5 μg/ml PI and 0.1 mg/ml RNase (DNase free) in PBS for 15 minutes at 37°C.[11] Sections were then washed with ddH_2O and coverslipped with Aqua-Poly/Mount (Polysciences). Stained sections were viewed using a rhodamine fluorescent filter.

Electron Microscopy

Eyes were fixed by intracardiac perfusion of 1% glutaraldehyde and 1% paraformaldehyde in PBS, pH 7.1. After perfusion, the eyes were enucleated, the anterior segment was removed, and the eyecups were immersed overnight in the aldehyde mixture. The specimens were washed in PBS, postfixed in veronal acetate-buffered osmium tetroxide (2%), dehydrated in a graded ethanol and water series, and embedded in Araldite (6005; Polysciences).

RESULTS

Analysis Using the TUNEL Technique

Positive controls, generated using DNase 1 in TdT buffer before incubation with terminal transferase and biotinylated nucleotides, showed 100% TUNEL-positive (T+) cells (Fig. 1B). Virtually no labeling of nuclei by the TUNEL method was seen in retinal sections from control animals (Fig. 1A). On the other hand, T+ cells were abundant 1 and 3 days after RD, when they were seen almost exclusively in the photoreceptor layer (Figs. 1C, 1D); they also were seen 7 days and, occasionally, 14 and 28 days after RD (not shown). TUNEL-positive cells always appeared isolated from each other, with no indications of aggregation into multicellular clusters. Because of the nature of the precipitate generated by the TUNEL technique, it was not possible to determine accurately whether apoptotic cells were present in the RPE (not shown).

Quantitative analysis verified this distinct temporal pattern (Fig. 2). Photoreceptor cell death occurred rapidly during the first 72 hours after RD, as indicated by a peak of T+ cells. Although there were occasional T+ nuclei in the ganglion cell layer and the inner nuclear layer, labeling of apoptotic nuclei was confined almost exclusively to the photoreceptor layer. The number of labeled apoptotic nuclei was much lower at all other time points studied.

Morphologic Analysis Using Propidium Iodide

Cells undergoing apoptosis typically have shrunken, highly condensed, and sometimes fragmented nuclei that stain more intensely with PI than do the nuclei of normal cells. Analysis of sections from control and RD retinas using this method yielded results similar to those obtained with the TUNEL technique; no PI-labeled pyknotic nuclei were seen in controls, whereas in RD retinas they were most abundant 1 to 3 days after RD and were found almost exclusively in the photoreceptor layer (Fig. 1E). Cell nuclei were visualized easily in the RPE layer using this method (Fig. 1F); however, no PI-labeled pyknotic nuclei were observed in control or experimental RD retinas.

Electron Microscopy

Apoptotic cells were clearly visible in a cat retina with an RD of 2 days duration, when it was possible to observe several degenerating cells in a single section (Fig. 3A). Electron microscopic signs of apoptosis included chromatin condensation and nuclear fragmentation (Fig. 3B), as well as "nuclear capping" (Fig 3C).

DISCUSSION

Our results can be summarized as follows: (1) Photoreceptor cell death after RD exhibits several of the characteristic landmarks of apoptosis, including light and electron microscopic evidence of pyknosis, as well as DNA fragmentation detectable with the TUNEL technique. (2) There is an earlier period of photoreceptor death than previously recognized; abundant T+ and PI+ cells can be detected as early as 1 to 3 days after RD, followed by a decline in their number over the next few weeks. (3) Within the retina, apoptotic cell death after RD largely appears to be limited to the outer nuclear layer. (4) Retinal pigment epithelial cells do not exhibit detectable signs of apoptotic cell death when photoreceptor death is extensive. We tried to visualize DNA ladders using agarose gel electrophoresis combined, in some cases, with DNA labeling and Southern transfer to increase sensitivity. Although some low-molecular-weight bands suggestive

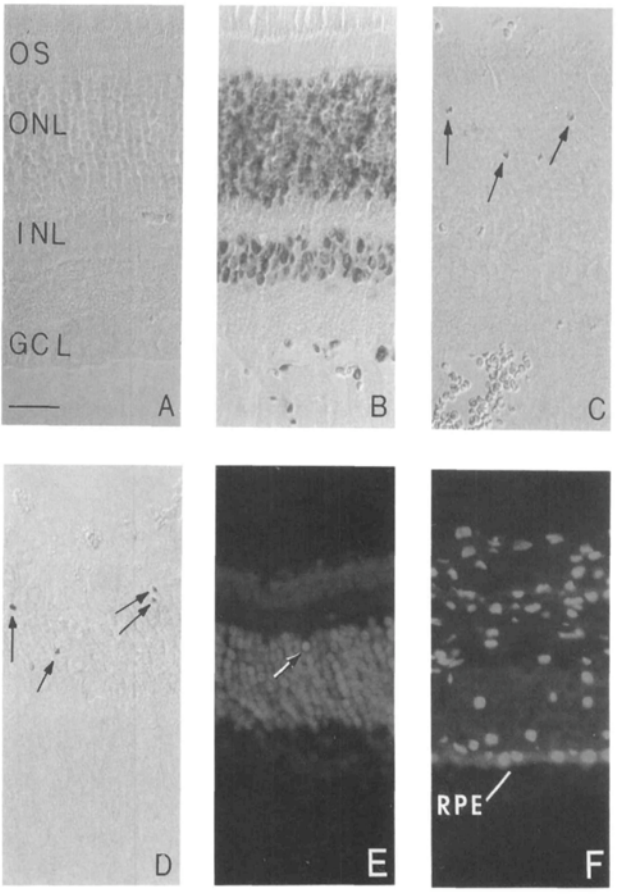

FIGURE 1. In situ retinal labeling by the TUNEL method (see Methods for details). (**A**) Control retina (from an adult cat with no retinal detachment); note the absence of TUNEL-labeled nuclei. (**B**) Positive control (retina from an adult cat with no retinal detachment, section treated with DNase I before being processed with the TUNEL technique); all nuclei are labeled. (**C**) Retina from an adult cat with a 1-day RD. Arrows indicate TUNEL-positive cells. (**D**) Retina from an adult cat with a 3-day RD. Arrows indicate TUNEL-positive cells in the ONL. (**E**) Propidium iodide-stained section of the retina from an adult cat with a 3-day RD, an intensely fluorescent cell is shown (*arrow*). (**F**) Propidium iodide-stained retinal pigment epithelium (*arrow*) from an adult cat with a 3-day RD. No pyknotic cells are seen. Calibration bar = 40 μm. TUNEL = terminal dUTP nick end labeling; OS = outer segments; ONL = outer nuclear layer; INL = inner nuclear layer; GCL = ganglion cell layer; RD = retinal detachment.

of internucleosomal fragments were observed in detached, but not in control, retinas (data not shown), the results were inconclusive, probably because of the small amounts of material available. Combined with the fact that virtually no T+ or PI+ cells were seen in unoperated control retinas, our findings are consistent with the hypothesis that cell death in the cat retina after RD occurs through an apoptotic mechanism and is largely restricted to photoreceptor cells.

At this time, we can only speculate about the mechanism responsible for photoreceptor death after RD. Given findings with other neuronal systems that show that trophic factor deprivation leads to apoptotic neuronal death,[29] it appears logical to propose that a similar phenomenon could be operative in RD, in which the subretinal space expands and the composition of the interphotoreceptor matrix (IPM) changes.[30] As RD persists, however, the IPM molecular composition could be restored because trophic agents are secreted and reaccumulate, which might reduce the rate of cell death. Trophic agents presumed to be present in the IPM include acidic fibroblast growth factor, basic fibroblast growth factor, transforming growth factor-α, and transforming growth factor-β,[31–37] as well as a photoreceptor-specific, survival-promoting macromolecular factor partially purified from IPM preparations.[38] The IPM is also rich in interphotoreceptor retinoid binding protein (IRBP), which facilitates the transport of retinoids between RPE and neural retina; the trophic role of retinoids has been documented in vivo[39] and in vitro.[40] Although other mechanisms must be considered, such as impaired transport of water and ions, the possible role of trophic factors in retinal detachment deserves

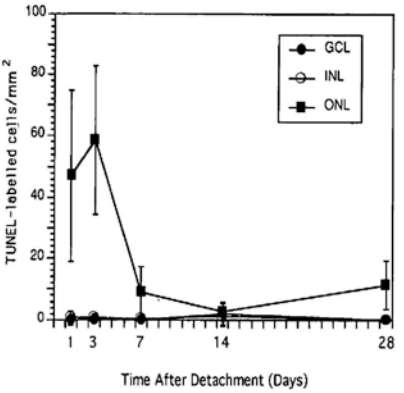

FIGURE 2. Quantitative analysis of apoptotic cell death in retinas at different stages after experimental retinal detachment. Three to four eyes were used for each time point, except for day 14, for which only two eyes were available. Approximately 25 sections per time point were processed with the TUNEL technique and were analyzed as described in Methods. Error bars represent standard deviation of the mean. Virtually no TUNEL-positive cells were seen in control retinas. TUNEL = terminal dUTP nick end labeling.

further investigation, particularly because of possible therapeutic applications.

The absence of apoptotic cell death in the RPE during stages when many photoreceptors are degenerating is of interest. A number of morphologic changes are known to occur in the cells of the RPE after RD.[17] Conspicuous among them is RPE proliferation, which in the cat retina begins by 24 hours after detachment and continues to be extensive by 48 to 72 hours. This was interpreted to indicate that close apposition of the RPE and the neural retina is a prerequisite for keeping the RPE in a mitotically inactive state. It appears, however, that such apposition is not essential for the survival of RPE cells.

Previous studies[17] reported an early decrease in photoreceptor number after RD, but this was attributed to edema (which would cause apparent decreases in photoreceptor density) rather than to actual photoreceptor losses. Our results, however, demonstrate a rapid wave of photoreceptor death soon after RD, which is already present by 1 day and (at least based on the stages included in this study) appears to peak on day 3. Previous research[18] suggests that rods degenerate more rapidly than cones, and the majority of the apoptotic cells at the peak of photoreceptor death are most likely rods. Some of the apoptotic cell bodies in RD sections (especially 3 days after RD) appear to be cones, however, because of their position adjacent to the outer limiting membrane. Electron microscopy of retinal sections at various time points reveals what appears to be both apoptotic rods and cones.[18] After the peak of cell death on day 3, the number of T+ cells appears to decline fairly rapidly because only few apoptotic cells are seen in retinas from animals with RD of 7 and 14 days duration; however, T+ cells are still seen in retinas from animals with detachments of 28 days duration. The mechanisms that bring about this apparent decrease in the speed of photoreceptor cell loss after RD remain unknown. As mentioned, it is possible that factors necessary for photoreceptor survival might reappear or increase in concentration in the subretinal space over time through the secretory contributions or RPE and retinal cells.

Although the present study did not investigate therapeutic approaches to RD, some general com-

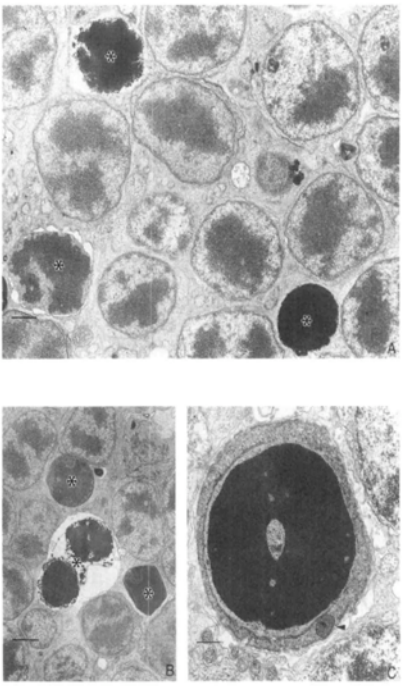

FIGURE 3. Electron micrographs of apoptotic cells from the retina of an adult cat with a 2-day retinal detachment. (**A**) Low-power magnification of the outer nuclear layer. Cells in different phases of apoptosis can be seen. Bar = 1.93 μm (**B**) High-power magnification of several cells in the outer nuclear layer undergoing apoptosis. One apoptotic cell appears to be undergoing nuclear fragmentation. Bar = 1.23 μm (**C**) High-power magnification of a single apoptotic cell in the outer nuclear layer demonstrating nuclear capping, a feature of apoptotic cells. Arrow points to a mitochondrion. Bar = 0.5 μm.

ments are warranted based on the timing and apoptotic nature of photoreceptor death in RD. The observation of extensive cell death shortly after RD is consistent with reports that detachments of short duration yield a better prognosis in the cat,[25] as well as in human patients.[41–44,45] Moreover, our findings suggest that the treatment modalities available for clinical retinal detachment[1,45,46] should be used with as little delay as possible to avoid the rapid wave of photoreceptor cell death that occurs in the retina after RD. Finally, our results suggest agents that interfere with the apoptotic mechanism itself may have a role in the prevention of cell death after RD. The rapid pace of progress in the molecular mechanisms of programmed cell death suggest that new therapeutic tools may become available in the not too distant future.

Key Words

apoptosis, retinal detachment, photoreceptor degeneration, retina, retinal degeneration

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