

# Caspase Activation in an Experimental Model of Retinal Detachment

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**PURPOSE.** To test for apoptotic photoreceptor cell death and caspase activation as a function of time after induction of an experimental retinal detachment.

**METHODS.** Retinal detachments were created in Brown Norway rats by injecting 10% hyaluronic acid into the subretinal space using a transvitreal approach. Light microscopy and terminal dUTP-biotin nick end-labeling (TUNEL) was performed at 1, 3, 5, and 7 days after detachment to assess for the morphologic features associated with apoptosis. Western blot analysis of retinal protein extracts was performed using antibodies against caspase-3, -7, and -9 and poly-ADP ribose-polymerase (PARP) at 1, 3, and 5 days after detachment.

**RESULTS.** Light microscopic analysis of detached retinas showed the presence of pyknotic nuclei in the outer nuclear layer and disruption of the normal organization of the photoreceptor outer segments. TUNEL-staining was positive in the outer nuclear layer only in the detached portions of the retina. Western blot analysis confirmed the time-dependent activation of caspase-3, -7, and -9 and PARP in the detached retinas. No morphologic stigmata of apoptosis or caspase activation was detected in attached retinas.

**CONCLUSIONS.** The apoptotic photoreceptor cell death in experimental retinal detachments is associated with caspase activation. (*Invest Ophthalmol Vis Sci.* 2003;44:1262-1267) DOI:10.1167/iovs.02-0492

Retinal detachments are a significant cause of vision loss, especially when they involve the central macula, and photoreceptor cell death is thought to be the major cause of this decrease in visual function.<sup>1</sup> In addition, structural and biochemical rearrangements that also contribute to vision loss occur in various retinal layers.<sup>2-7</sup>

Apoptosis—programmed cell death—mediates photoreceptor cell death during retinal detachments.<sup>8-11</sup> Apoptosis involves the orderly breakdown and packaging of cellular components and their subsequent removal by surrounding structures.<sup>12</sup> In general, apoptosis does not result in the activation of an inflammatory response. This is in contrast to necrotic cell death, which is characterized by the random

breakdown of cells in the setting of an inflammatory response. Detecting apoptosis involves assaying for the morphologic and biochemical stigmata associated with cellular breakdown and packaging, such as pyknotic nuclei, apoptotic bodies (vesicles containing degraded cell components) and internucleosomally cleaved DNA. This last feature is specifically detected by binding and labeling the exposed 3'-OH groups of the cleaved DNA with the enzyme terminal deoxynucleotidyl transferase (TUNEL assay). After apoptosis has been initiated, a complex series of second messengers and cell-death-specific proteins become activated. One such group of proteins is a family of serine-proteases known as caspases.<sup>12</sup> There are approximately 13 known caspases, and activation of these proteins results in the proteolytic digestion of the cell and its contents. The presence of multiple caspase proteins and the transduction cascade required for their activation allows for multiple levels of control of apoptosis. Detecting caspase activation is one method by which the presence of apoptotic cell death can be measured.

Apoptotic photoreceptor cell death has been examined in cat<sup>8</sup> and rat<sup>11</sup> models of experimental retinal detachments. These investigators have shown the presence of the stigmata of apoptosis, including the presence of pyknosis, apoptotic bodies, and internucleosomally cleaved DNA. In addition, Hisatomi et al.<sup>11</sup> showed the cellular redistribution of apoptosis-inducing factor (AIF, an apoptosis regulatory protein) during retinal detachments. In this study we demonstrated apoptotic photoreceptor cell death and time-dependent activation of caspase-3, -7, and -9 and the cleavage of poly-ADP ribose-polymerase (PARP) in a rat model of experimental retinal detachment.

## MATERIAL AND METHODS

### Retinal Detachments

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines established by the Animal Care Committee of the Massachusetts Eye and Ear Infirmary. Adult male Brown Norway rats (300–450 g, Charles River, Boston, MA) were used in this study. Retinal detachments were created by using a variation of the previously described models of experimental retinal detachments.<sup>8,11</sup> Rats were anesthetized with a 50:50 mixture of ketamine (100 mg/mL) and xylazine (20 mg/mL; both from Phoenix Pharmaceutical, St. Joseph, MO). Pupils were dilated with a topically applied mixture of phenylephrine (5.0%) and tropicamide (0.8%; Massachusetts Eye and Ear Infirmary internal formulary preparation). A sclerotomy was created approximately 2 mm posterior to the limbus with a 20-gauge microvitreoretinal blade (MVR; BD Biosciences, Franklin Lakes, NJ). Care was taken not to damage the lens during creation of the sclerotomy. A Glaser subretinal injector (20-gauge shaft with a 32-gauge tip, BD Biosciences) connected to a syringe filled with 10 mg/mL sodium hyaluronate (Healon; Pharmacia and Upjohn Co., Kalamazoo, MI) was then introduced into the vitreous cavity. A retinotomy was created in the peripheral retina with the tip of the subretinal injector, and the sodium hyaluronate was slowly injected into the subretinal space, thus detaching the retina from the underlying RPE. Retinal detachments

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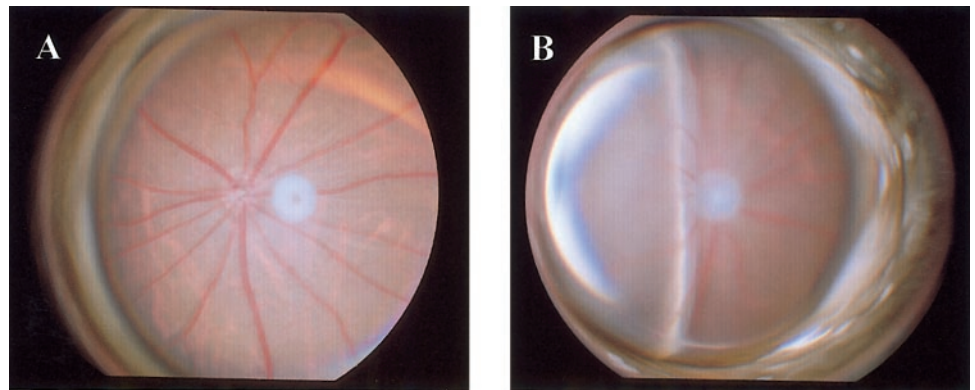
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**FIGURE 1.** (A) Fundus photograph of attached rat retina. The *white ring* adjacent to the optic nerve is a flash artifact. (B) Fundus photograph of the retina shown in (A) after creation of the retinal detachment. The detachment extends counterclockwise from approximately 11:30 to 6:30.

were created only in the left eye of each animal, with the right eye serving as the control. In each experimental eye, approximately one half of the retina was detached, allowing the attached portion to serve as a further control.

### Histology and TUNEL-Staining

Eyes were enucleated at 1, 3, 5, and 7 days after creation of the retinal detachment. Enucleation was performed with the anesthesia used for the detachments. For light microscopic analysis the cornea and lens were removed, and the remaining eyecup was placed in a fixative containing 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C overnight. Tissue samples were then postfixed in 2% osmium tetroxide, dehydrated in graded ethanol, and embedded in epoxy resin. One-micrometer sections were stained with 0.5% toluidine blue in 0.1% borate buffer and examined with a photomicroscope (Axiophot; Carl Zeiss, Oberkochen, Germany).

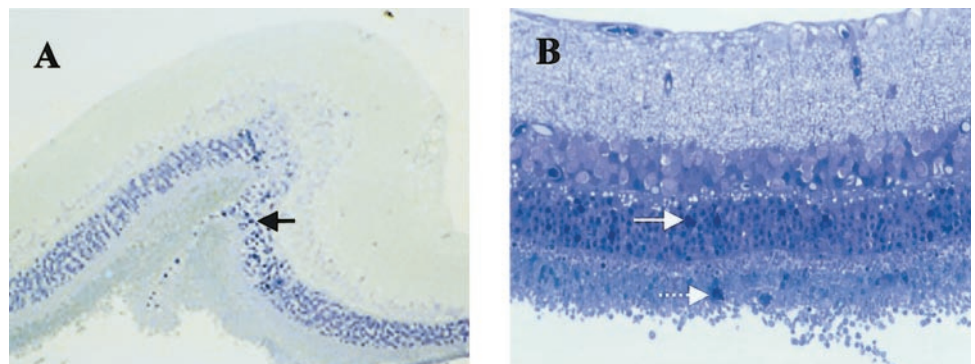
For TUNEL staining the cornea and lens were not removed after enucleation, but rather the whole eye was fixed overnight at 4°C in a solution of phosphate-buffered saline with 4% paraformaldehyde (pH 7.4). A section was then removed from the superior aspect of the globe, and the remaining eyecup was embedded in paraffin and sectioned at a thickness of 6  $\mu$ m. TUNEL staining was performed on these sections with a kit (TdT-Fragel DNA Fragmentation Detection Kit; Oncogene, Boston, MA) according to the manufacturer's instructions. Reaction signals were amplified with a preformed avidin-biotinylated enzyme complex (ABC kit; Vector Laboratories, Burlingame, CA). Internucleosomally cleaved DNA fragments were stained with diaminobenzidine (DAB; staining indicates TUNEL-positive cells), and sections were then counterstained with methylene green.

### Western Blot Analysis

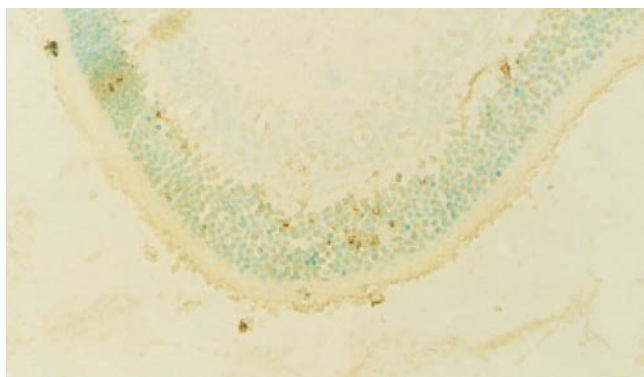
For Western blot analysis, retinas from both experimental and control eyes were manually separated from the RPE-choroid at days 1, 3, and 5

after creation of the retinal detachment. In eyes with retinal detachments, the experimentally detached portion of the retina was separated from the attached portion of the retina and analyzed separately. Retinas were homogenized and lysed with buffer containing 1 mM EDTA-EGTA-dithiothreitol (DTT), 10 mM HEPES (pH 7.6), 0.5% IGEPAL, 42 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM PMSF, and 1 tablet of protease inhibitors per 10 mL buffer (Complete Mini; Roche Diagnostics GmbH, Mannheim, Germany). Samples were incubated for 15 minutes on ice, and then centrifuged at 21,000 rpm at 4°C for 30 minutes. The protein concentration of the supernatant was determined with the reagents in a kit (D<sub>c</sub> Protein Assay; Bio-Rad Laboratories, Hercules, CA). Proteins were separated on SDS-PAGE gels (7.5% and 15% Tris-HCl Ready-Gels; Bio-Rad Laboratories), 30  $\mu$ g of total retinal protein per lane, transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA), and blocked with 5% nonfat dry milk in 0.1% TBS-T. Membranes were incubated with antibodies against caspase-7 (1:1000; Cell Signaling Technology, Beverly, MA), caspase-9 (1:1000; Medical & Biological Laboratories, Naka-ku Nagoya, Japan), cleaved caspase-3 (1:1000; Cell Signaling Technology), caspase-3 (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA), or PARP (1:1000; Cell Signaling Technologies) overnight at 4°C. Bands were detected using the enhanced chemiluminescence reagent (ECL-Plus; Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were exposed to autoradiographic film (HyperFilm; Amersham), and densitometry was performed on computer (ImageQuant 1.2 software; Molecular Dynamics, Inc., Sunnyvale, CA). For each eye tested, densitometry levels were normalized by calculating the ratio of the cleaved form to the pro form of the protein of interest. Procaspase-7 levels were normalized to the densitometry readings from a nonspecific band detected by the secondary IgG. Five eyes were used for each time point, except for the PARP levels for day 5 after detachment for which only four eyes were used. All statistical comparisons were performed with a paired *t*-test.

**FIGURE 2.** (A) Light microscopic section of a detached retina at 1 day after creation of the retinal detachment. This section shows photoreceptor cells pushed along the tract made through the retina by the subretinal injector. Pyknotic photoreceptor cell nuclei appeared only at the edge of the injection site (*black arrow*). (B) Light microscopic section of a retina detached for 3 days. More abundant pyknosis was seen in the outer nuclear layer (*solid white arrow*), with extrusion of one of these pyknotic nuclei out through the outer segment layer (*dashed white arrow*). Note the marked disruption of the normal orderly array of the photoreceptor outer segments. No pyknosis was observed in any of the inner retinal layer cells. Toluidine blue. Magnification: (A)  $\times 30$ ; (B)  $\times 40$ .



Note the marked disruption of the normal orderly array of the photoreceptor outer segments. No pyknosis was observed in any of the inner retinal layer cells. Toluidine blue. Magnification: (A)  $\times 30$ ; (B)  $\times 40$ .



**FIGURE 3.** TUNEL staining of a paraffin-embedded section of a retina detached for 3 days. *Dark brown* nuclei represent TUNEL-positive staining. DAB with methylene green counterstain. Magnification,  $\times 40$ .

## RESULTS

Figure 1 shows a typical example of a rat retina after creation of the experimental detachment. Intraocular pressures were measured before and immediately after detachment with a handheld tonometer (Tono-Pen; Mentor, Norwell, MA) and no difference was found (data not shown).<sup>13</sup> The retinal break created by the subretinal injector was confined to the site of the injection. Occasionally, a small, but always minimal, hemorrhage (less than two to three disc areas) occurred at the site, localized to the site of the injection. Because of the large size of the lens compared with the total volume of the eye the maximum height of the detachments was generally limited to 2 or 3 mm. Occasionally, the retina adhered to the posterior surface of the lens. After the creation of the detachment, the sodium hyaluronate remained in the subretinal space although the maximal height of the detachments decreased slowly as a function of time, probably because of reabsorption of water from the subretinal solution of sodium hyaluronate.

Light microscopic analysis of detached retinas shows an increase in morphologic stigmata of apoptosis as a function of time after detachment. At 1 day after creation of the detachment, pyknosis in the outer nuclear layer was confined to the area of the peripheral retinotomy site through which the subretinal injector was introduced (Fig. 2A). By day 3, however, there were pyknotic nuclei in the whole outer nuclear layer in the area of the detachment (Fig. 2B), extruding from the outer nuclear layer into the subretinal space. The remaining layers of the retina appeared morphologically normal. No inflammatory cells were seen, and there was no apparent disruption of the retinal vasculature. Similar changes were noted in sections from retinas detached for up to 1 week. No pyknotic nuclei were detected in the area of the attached retina or in the fellow

eye with nondetached retina. The amount of outer nuclear layer pyknosis was similar between detachments of 3 day's or 1 week's duration.

Disruption of the photoreceptor outer segments was a prominent feature in the detached retinas. Outer segments of the control eyes and the attached portions of the experimental eyes had an orderly, parallel arrangement. Detachments produced artifactually during tissue processing in these eyes did not alter the photoreceptor morphology. In contrast, the photoreceptor outer segments of detached retinas were severely disorganized and lost their normal structural organization. In addition, outer segments in attached areas had similar lengths, whereas the outer segments in detached areas showed variable lengths.

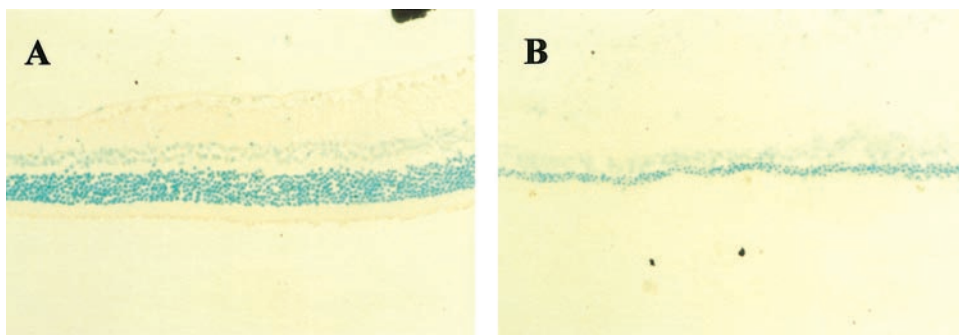
Internucleosomal DNA cleavage in photoreceptor cells was detected with the TUNEL assay. TUNEL-positive cells were detected at all time points tested (1, 3, 5 and 7 days after detachment; Fig. 3) and was confined to the photoreceptor cell layer. We continued to observe two eyes, and retinal detachment persisted for 2 months. The TUNEL assay at 2 months did not reveal any staining indicating the presence of internucleosomally cleaved DNA. Of note, this prolonged detachment was associated with a marked reduction in the thickness of and number of cell bodies contained in the outer nuclear layer compared with the nondetached retina (Fig. 4).

Antibodies specific for caspase-3, -7, and -9 and PARP were used to probe total retinal protein extracts at various times after creation of the retinal detachment. The cleaved, or active, form of caspase-3 was elevated in the detached retinas compared with the attached retinas. The level of cleaved caspase-3 increased as a function of time after detachment, with a peak at approximately 3 days (Fig. 5A). No cleaved caspase-3 was detected in the control eye or in the attached portion of the retina in the experimental eye.

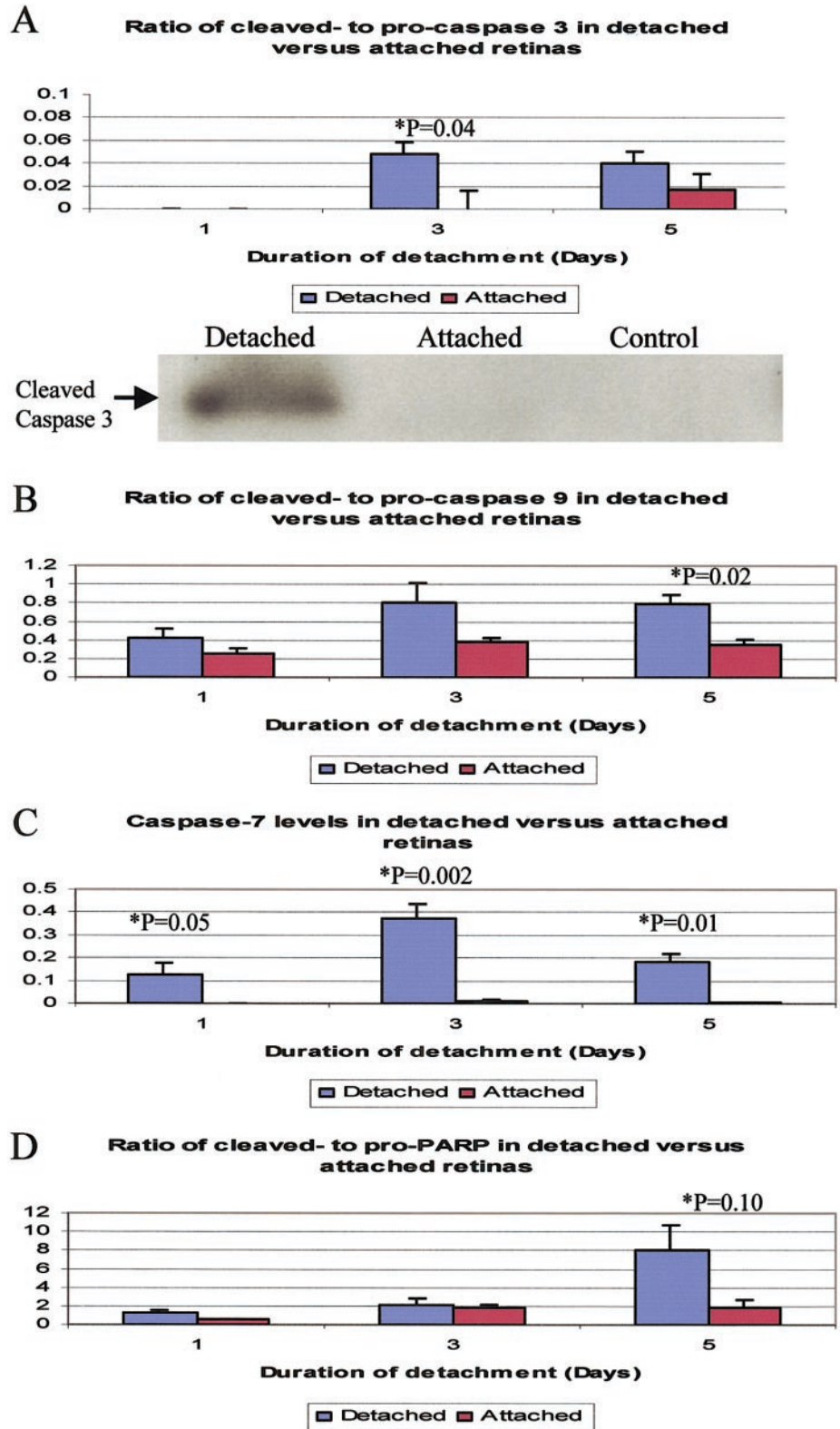
The ratio of the active to inactive form of caspase-9 also increased as a function of time after creation of the experimental retinal detachment (Fig. 5B). The peak level of cleaved caspase-9 was seen at 3 to 5 days after creation of the detachment. The caspase-7 antibody was able to detect only the pro form of the protein. There was, however, a significant difference in the amount of the pro form detected in the protein extract from the detached retinas compared with the attached retinas (Fig. 5C). Western blot analysis with antibodies against PARP—a component of the apoptosis cascade downstream of caspase-7—detected an increase in the level of cleaved PARP that was at its maximum at 5 days after detachment (Fig. 5D). Probabilities for the comparisons between detached and attached retinas are shown in Figure 5.

## DISCUSSION

Apoptosis has been implicated in photoreceptor cell death after retinal detachments by several investigators,<sup>8-10</sup> who



**FIGURE 4.** (A) Control, nondetached, retina from the eye of a rat with a 2-month detachment of the retina in the other eye. (B) Light microscopic section of a retina detached for 2 months. Note the markedly thinned outer nuclear layer compared with the control eye shown in (A). DAB with methylene green counterstain. Magnification,  $\times 20$ .



**FIGURE 5.** (A) Ratio of the cleaved to pro form of caspase-3 as a function of time after detachment of the retina. The Western blot below the chart shows the band for cleaved caspase-3 at 3 days after creation of the detachment. Note the increased band density in the detached retina compared with the control eye or the attached portion of the experimental eye. (B) Ratio of cleaved to procaspase-9 as a function of time after detachment. (C) Caspase-7 levels as a function of time after detachment. (D) Ratio of cleaved to pro-PARP as a function of time after detachment. \*Statistical significance at the probability shown. Five eyes were used for each time point, except for the PARP levels on day 5 after detachment, for which only four eyes were used. All statistical comparisons were performed with a paired *t*-test. The ordinate represents relative densitometry units.

demonstrated the presence of the various stigmata associated with apoptotic cell death in both human and experimental retinal detachments. Microscopic analysis in these studies showed the presence of pyknotic nuclei and apoptotic bodies, and TUNEL staining was positive for the presence of internucleosomal cleaved DNA. In addition, the photoreceptors of detached retinas showed a reorganization of their cytoskeleton

consistent with cell death.<sup>5</sup> Retinal detachments have also been shown to induce local increases in the levels of excitotoxic amino acids in the microenvironment surrounding photoreceptors. Neighboring cells such as Müller cells and retinal pigment epithelial cells attempt to sequester these amino acids, presumably in an effort to minimize the induction of apoptosis by these toxic factors.<sup>7</sup>

The model of retinal detachment described herein shows histologic characteristics consistent with the previously described models and with the activation of apoptosis in the photoreceptor cells.<sup>8,11</sup> No evidence of necrotic cell death was seen in our model. Furthermore, the timing of the appearance of the stigmata of apoptosis in our model is consistent with the timing in the other models. The activation of apoptosis in our model was confined to the photoreceptor cell layer, suggesting that the separation of the photoreceptor cells from the retinal pigment epithelium preferentially affects the outer retina.

The correlation of our animal model of retinal detachments with rhegmatogenous retinal detachments in humans should be defined. Similar to previously described models of experimental retinal detachment,<sup>8,11</sup> our model uses a 10% hyaluronic acid solution to create the retinal detachments. The subretinal fluid in human detachments contains hyaluronic acid<sup>14,15</sup> but at a much lower concentration (<1%).<sup>16</sup> The effect of this difference on a variety of factors such as oxygen or nutritional diffusion from the choroid to the photoreceptors is unclear.

Not all photoreceptor cells in our model demonstrated morphologic evidence of apoptosis after detachment. The choroid and retinal pigment epithelium provide many metabolic functions for the photoreceptor cells, and it might be expected that disruption of these functions would be equally damaging to all the photoreceptor cells. The data suggest that apoptosis proceeds in a subpopulation of cells and eventually leads to substantial cumulative loss. What triggers certain cells to embark on apoptosis and others to be protected for a time is as yet unknown. The chronic detachment, however, demonstrates a severe reduction in the number of photoreceptor cells by 2 months. This time lag between detachment and morphologic thinning of the retina suggests cumulative cell death from prolonged separation of the photoreceptor cells from their normal anatomic position. It also suggests the presence of a protective mechanism that provides a window of opportunity for reattaching the retina before widespread damage can occur. However, the specific duration of this window and the rate of photoreceptor attrition during retinal detachment still remain unknown.

Caspase activation plays an important part in the transduction pathway of the apoptosis cascade, and has been implicated in a number of other ocular diseases.<sup>17-19</sup> The role of caspase activation in photoreceptor cell death in retinal disease is controversial and may depend on the specific disease being studied and the model being used to study that disease. Models in which photic injury is used to induce retinal degeneration may cause photoreceptor cell death through caspase-independent pathways.<sup>20</sup> In contrast, models that use a chemical induction of photoreceptor degeneration show that caspase activation occurs and that caspase inhibitors can decrease the amount of cell death.<sup>21,22</sup>

Our work is the first to show the activation of caspases, particularly caspase-3, -7, and -9, in an experimental model of retinal detachments. This is in contrast to the conclusions of Hisatomi et al.,<sup>11</sup> who suggested that photoreceptor apoptosis in retinal detachment did not require activation of caspase. They based this conclusion on their observation that TUNEL staining, relocalization of AIF, and relocalization of cytochrome-*c* occurs even in the presence of the caspase inhibitor Z-VAD.fmk. They also noted the presence of these apoptosis markers in animals with a nonfunctional CD95/CD95-ligand system. These assays, however, are measures of apoptosis and not necessarily of caspase function. In fact, the CD95/CD95-ligand system is only one possible receptor mechanism for activating the apoptosis cascade. Perhaps in these animals, or in retinal detachments in general, another receptor is responsible for the activation of apoptosis. In addition, the inhibitor

used in their experiments may not have penetrated the target tissues or may have been at a dose insufficient to inhibit apoptosis adequately.

Our work provides a direct measurement of the degree of caspase activation. Although our Western blot analyses were run on extracts of total retinal proteins, the presence of pyknosis and TUNEL-positive staining only in the photoreceptor cell layer suggests that the caspase activation results from the initiation of apoptosis in these cells only.

Modulation of the apoptotic cascade has been shown to alter the rate and amount of photoreceptor cell death in a variety of models of retinal degeneration.<sup>23-29</sup> A protective effect on the photoreceptor cells has also been demonstrated in experimental retinal detachments by the addition of brain-derived neurotrophic factor<sup>11,30</sup> and by the administration of 100% oxygen to animals with retinal detachments.<sup>31,32</sup> Our findings suggest that interventions to prevent activation of caspase may provide a new avenue for further research in therapy for retinal disorders. These interventions may have utility in many clinical situations in which the microenvironment of the neural retina is disturbed, including rhegmatogenous retinal detachments, exudative macular degeneration, or tumors.

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