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Relocalization of Apoptosis-Inducing Factor in Photoreceptor Apoptosis Induced by Retinal Detachment *in Vivo*

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Apoptosis-inducing factor (AIF) is a novel mediator in apoptosis. AIF is a flavoprotein that is normally confined to the mitochondrial intermembrane space, yet translocates to the nucleus in several *in vitro* models of apoptosis. To investigate the role of AIF in the apoptotic process *in vivo*, we induced retinal detachment (RD) by subretinal injection of sodium hyaluronate, either in Brown Norway rats or in C3H mice. Apoptotic DNA fragmentation, as determined by terminal nick-end labeling, was most prominent 3 days after RD. The subcellular localization of AIF was examined by immunohistochemistry and immunoelectron microscopy. In normal photoreceptor cells, AIF was present in the mitochondrion-rich inner segment. However, AIF was found in the nucleus after RD. Photoreceptor apoptosis developed similarly in C3H control mice, and in mice bearing the gld or lpr mutations, indicating that cell death occurs independently from the CD95/CD95 ligand system. Both the mitochondrio-nuclear transition of AIF localization and the nuclear DNA fragmentation were inhibited by subretinal application of brain-derived neurotrophic factor. To our knowledge, this is the first description of AIF relocalization occurring in a clinically relevant, *in vivo* model of apoptosis. *(Am J Pathol 2001, 158:1271–1278)*

Apoptosis plays a critical role in biological processes such as embryogenesis, immunogenesis, and carcinogenesis.[1,2] Recently it has become widely accepted that mitochondria play a key role in the control of apoptotic cell death.[3–5] Early during the apoptotic process, mitochondria can release several apoptogenic proteins including cytochrome *c*; procaspase-2, -3, and -9; as well as apoptosis-inducing factor (AIF), through the outer mitochondrial membrane into the cytosol. The relocalization of such apoptogenic proteins occurs when apoptotic signals including pro-apoptotic members of the Bcl-2 family induce opening of the permeability transition pore and/or induce the formation of protein-permeant conduits in the outer mitochondrial membrane. Loss of mitochondrial membrane integrity may also affect the mitochondrial electron transport, induce an enhanced generation of reactive oxygen species, and compromise the generation of ATP.[4,6–8]

AIF is a novel, caspase-independent apoptogenic factor. In the absence of apoptotic signals, AIF is normally confined to the mitochondrial intermembrane space.[9–11] However, when apoptosis is induced in HeLa cells, Rat-1, or Jurkat cells *in vitro,* AIF translocates to the cytosol and to the nucleus.[9,10,12,13] When recombinant AIF is injected into the cytoplasm of intact cells or added to purified nuclei from HeLa cells, it causes the nucleus to undergo peripheral chromatin condensation and triggers large-scale DNA degradation to fragments of ~50 kbp.[9] AIF also induces purified mitochondria to release cytochrome *c* and caspase-9, suggesting that AIF, once released from mitochondria, accelerates membrane permeabilization in a positive feedforward loop.[9] Microinjection of AIF into the cytoplasm of the intact cells induced a loss of the mitochondrial transmembrane potential and the exposure of phosphatidylserine on the surface of the plasma membrane.[9] All these changes occur in the presence of saturating doses of the wide-ranging caspase inhibitor Z-VAD.fmk, indicating that AIF acts in a caspase-independent manner.

AIF is strongly conserved among mammalian species (>95% amino acid identity between mouse and human) and bears a highly significant homology with flavoprotein oxidoreductases from all eukaryotic and prokaryotic kingdoms in its C-terminal portion.[10] Based on these findings, it is reasonable to speculate that AIF may be a phyloge-

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netically old mediator participating in various aspects of the apoptotic process. However, all studies supporting this hypothesis have been performed *in vitro.* The role of AIF in apoptosis *in vivo,* especially in mammalian pathology, remains unknown.

Retinal photoreceptors are neuroectodermal cells essential for vision.[14] A specific part of these polar cells, the ellipsoid, within the inner segment, is packed with regularly arranged mitochondria. Photoreceptors degenerate on traumatic or spontaneous retinal detachment (RD), which is one of the common causes of legal blindness in the young adult. Cell loss is reported to be because of apoptosis rather than because of necrosis.[15–19] Given the fact that RD usually occurs without inflammation or destructive ischemia, it provides a suitable context for studying the morphological changes involved in apoptosis. In this study, we assessed the possible role of AIF in photoreceptor apoptosis induced by experimental RD. Our data provide the first description of a subcellular relocalization of AIF *in vivo.*

Materials and Methods

Detection of Apoptosis

DNA Nick-End Labeling by the TUNEL Method

Apoptotic photoreceptor degeneration was detected by TdT-dUTP terminal nick-end labeling (TUNEL). Four-μm-thick sections were made from samples fixed in 4% paraformaldehyde and embedded in paraffin. TUNEL staining was performed with the ApopTag fluorescein direct *in situ* apoptosis detection kit (Intergen Company, New York, NY) according to the manufacturer's protocols. The sections were co-stained with propidium iodide (Molecular Probes, Eugene, OR), allowing observation of the cell nuclei by a fluorescence microscope (Olympus, Tokyo, Japan). Because the number of photoreceptors in each slide varied depending on the cutting angle, the number of apoptotic photoreceptors also varied. To avoid this sampling artifact, the rate of apoptotic photoreceptors was calculated using the following formula: apoptotic photoreceptor ratio (%) = total number of TUNEL-positive photoreceptors/total number of photoreceptors in the section. Ten sections for each eye specimen were randomly selected and observed by masked observers (six eyes for each time point).

AIF and Glial Fibrillary Acidic Protein (GFAP) Immunohistochemistry

Samples were fixed in 4% paraformaldehyde, embedded in paraffin, deparaffinized in xylene, rehydrated in ethanol, and washed in phosphate-buffered saline (PBS), as described above. A 1:100 dilution of anti-AIF rabbit serum was produced by a previously described method[9] and incubated at 4°C overnight. A nonimmune serum and a pre-absorbed antiserum (with 1 μg/μl recombinant AIF) were used as negative controls. Cy5-labeled secondary antibody (Zymed Laboratories, San Francisco, CA) was used at a dilution of 1:200 for 20 minutes. The sections were co-stained by TUNEL and observed with a fluorescence microscope. Furthermore, to clarify the localization of AIF in photoreceptors or Müller cells, a double immunostaining was made for Müller cell marker, GFAP (1:100 dilution, Santa Cruz Biotechnology, CA) and Cy5-labeled secondary antibody (KPL, Gaithersburg, MD).

Cytochrome c Immunohistochemistry

Four-μm-thick sections were made from samples fixed in 4% paraformaldehyde and embedded in paraffin. Anti-cytochrome *c* antibody (PharMingen, St. Louis, MO) was used at a 1:200 dilution and the sections were incubated at 4°C overnight. Cy5-labeled secondary antibody (KPL, Gaithersburg, MD) was used at a dilution of 1:200 for 20 minutes and the sections were observed with a fluorescence microscope.

Electron Microscopy and Immunoelectron Microscopy

The eyes were enucleated and the posterior segments were fixed in 1% glutaraldehyde and 1% paraformaldehyde in PBS. The detached retinas were removed and postfixed in veronal acetate buffer osmium tetroxide (2%), dehydrated in ethanol and water, and embedded in Epon. Ultrathin sections were cut from blocks and mounted on copper grids. For immunoelectron microscopy, the eyes were fixed in 1% paraformaldehyde in PBS, and the detached retinas were rinsed with PBS, incubated in NH_4Cl, and embedded in London Resin white blocks (London Resin, London, UK).[20] Primary antibody for AIF was used at a 1:150 dilution, and the sections were incubated at 4°C overnight. Anti-rabbit antibody conjugated with 10 nm gold particles (British BioCell, Cardiff, UK) was used as a secondary antibody at a dilution of 1:30 for 90 minutes. The specimens were observed with a JEM 100CX electron microscope (JEOL, Tokyo, Japan). A nonimmune serum was used as a negative control.

Experimental RD

All procedures conformed to the standards set forth in the Association for Research in Vision and Ophthalmology's statement for the Use of Animals in Ophthalmic and Vision Research. Brown Norway rats (Kyudo, Fukuoka, Japan), postnatal 8 weeks, were studied as follows.

The rats were anesthetized with an intraperitoneal injection of pentobarbital and their pupils were dilated with topical 1% tropicamide and 2.5% phenylephrine hydrochloride. The retinas were detached using a subretinal injection of 1% sodium hyaluronate (Pharmacia, Uppsala, Sweden). An anterior chamber puncture was performed from the corneal limbus to lower the intraocular pressure. Sclera was penetrated at the ocular nasal equator with a 30G needle, and then the needle was slowly advanced into the vitreous space. Soft retina was focally detached from comparatively hard retinal pigment epithelium as the needle moved. Then the needle was pulled and ad-

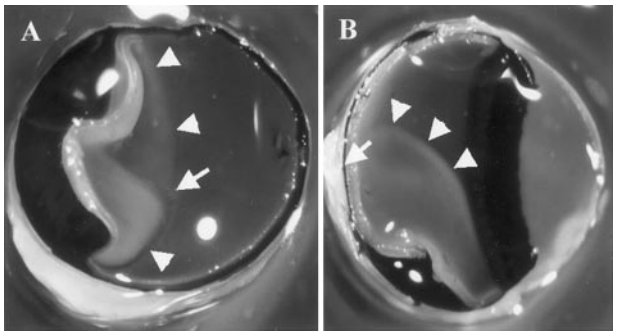

Figure 1. Rat eyes with RD. The eyes were fixed in 1% glutaraldehyde and 1% paraformaldehyde in PBS, cut in half, and the lens removed. Note the grayish detached retina (**arrowheads**). The **arrow** indicates the optic nerve head. **A:** A frontal section observed from the anterior side. **B:** A sagittal section.

vanced into the subretinal space beneath the focally detached retina. Sodium hyaluronate (0.05 ml) was injected gently to enlarge the RD. Because the volume of the vitreous space of the rat eye is relatively small, the injection of 0.05 ml of sodium hyaluronate reproducibly produced similar RDs (half of the retina, Figure 1). Biomicroscopy (Kowa, Tokyo) demonstrated that the same portion of the retina remained detached for more than 28 days after treatment. Detached retinas that reattached spontaneously were excluded from further study. Eyes with anterior chamber puncture, scleral incision, and no injection of sodium hyaluronate were used as controls. The rats were sacrificed at 6 and 12 hours and on days 1, 3, 5, 7, 14, and 28 after treatment and their eyes were harvested for the following studies. To evaluate the change of outer nuclear layer thickness after RD, the thickness of outer nuclear layer in histological sections (10 sections each day) was measured and analyzed using analysis software (MacScope; Mitani, Fukui, Japan). The results were expressed as the percentage of nondetached control retina.

CD95/CD-95 Ligand System, Downstream Caspases, and AIF in RD

To investigate the role of CD95/CD-95 ligand system in apoptosis, RDs were created in CD95/CD-95 ligand gene mutation mice, C3H-lpr and C3H-gld mice (SLC, Shizuoka, Japan), postnatal age 8 weeks using the method just described. C3H-lpr mice, a model of lymphoproliferation with CD95 gene mutation, and C3H-gld mice, a model of generalized lymphoproliferative disease with CD95 ligand gene mutation have been shown to lack functional CD95 and CD95 ligand.[21–27] Almost the entire retina was detached in these animals and remained detached. Our preliminary study showed that C3H wild-type mice showed a similar apoptotic time course as Brown Norway rats did, and apoptosis was evaluated on a representative day after detachment (day 3). Furthermore, to investigate the participation of caspases, RD was created in rats with sodium hyaluronate containing the wide-ranging caspase inhibitor, Z-VAD.fmk (25 ng per eye).

Neurotrophic Factor and AIF in RD

It is known that the outer layers of the retina containing the photoreceptor cells are supplied by diffusion from the choriocapillaris. To investigate whether apoptosis in RD and AIF depend on nutrition-dependent pathways, RDs were created with sodium hyaluronate containing a representative neurotrophic factor, brain-derived neurotrophic factor (BDNF, 2.5 ng per eye). BDNF and trkB expression have been found in ocular tissue,[28,29] and BDNF became a particularly interesting candidate for trophic activity, especially in the retinal pigment epithelium (RPE), because of its inducibility,[30] and its ability of photoreceptor-protection from degeneration.[31,32]

Results

Apoptosis in Experimental RD

After injection of sodium hyaluronate into the subretinal space of Brown Norway rats, RD was subjected to a macroscopic and histological follow-up for 28 days. In macroscopic terms, the surface of the detached area remained unchanged during this period (Figure 1). Histopathological examination revealed that the detached retinas degenerated gradually throughout time, progressively losing photoreceptors. No inflammatory cell infiltration was noted. Whereas control retinas do not label with TUNEL, on RD, TUNEL-positive apoptotic cells appeared in the photoreceptors of the outer retinal layer starting 12 hours after RD, reached a maximum on day 3, and then gradually diminished (Figure 2 and Figure 3). Simultaneously, the outer nuclear layer thickness of the retina, representing the photoreceptor layer decreased gradually to 88% of the control retina on day 3, 60% on day 7, and 32% on day 28.

AIF, GFAP, and Cytochrome c in Experimental RD

Immunohistochemistry showed specific AIF staining in ganglion cells, inner nuclear layer, outer plexiform layer and photoreceptors (Figure 4A, arrows). Immunostaining of control retinas revealed a multilinear pattern of AIF (Figure 4A, arrowheads) within the inner segment of photoreceptors, that is the mitochondrion-rich portion of the cells. This AIF-staining pattern did not change during the first 6 hours after RD. However, 12 hours after RD, we observed in some photoreceptors that AIF distributed in a diffuse manner throughout the cytosol and the nucleus (Figure 4B, arrows). No positive staining was found either by nonimmune serum or a pre-absorbed antiserum (with 1 μg/μl recombinant AIF; Figure 4, C and D). The double immunostaining showed that AIF was not localized in the GFAP-positive cells (Figure 4E). On the other hand, a double staining for AIF and TUNEL experiments revealed that AIF-positive nuclei from photoreceptor cells mostly are TUNEL-positive, at least during the first days after RD (Figure 5 and Table 1). Electron microscopy confirmed RD-induced morphological alterations in the photorecep-

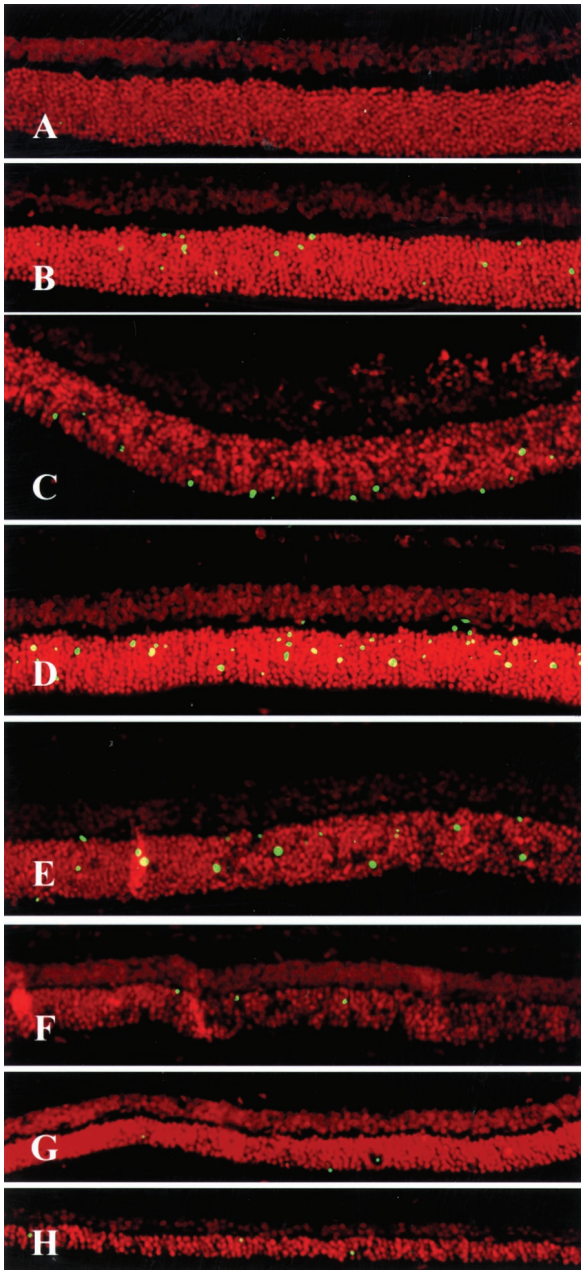

Figure 2. Fluorescent micrographs by TUNEL of sections from experimental RD from 6 hours to 28 days after treatment. Each section was stained with TdT-dUTP TUNEL and propidium iodide (see Materials and Methods). Note the TUNEL-positive cells in the photoreceptor layer (green). The highest frequency of TUNEL-positive photoreceptors is observed 3 days after treatment. The thickness of the outer nuclear layer decreases throughout time. **A:** 6 hours after treatment; **B**, 12 hours; **C**, 1 day, **D**, 3 days; **E**, 5 days; **F**, 7 days; **G**, 14 days; **H**, 28 days. Original magnifications, ×200.

tor layer, including chromatin condensation and cell shrinkage (Figure 6, A–E). Photoreceptors from control retinas contain AIF in their mitochondria but not in the nucleus, as revealed by immunogold electron microscopy (Figures 7 and 8). In contrast, dense AIF staining of the nucleus was observed in apoptotic cells, 3 days after RD (Figure 7). Thinning of the outer nuclear layer because of loss of photoreceptors (Figure 2, F–H) and deconstruction of the inner and outer segments of the photoreceptors were also observed (Figure 8, A and B).

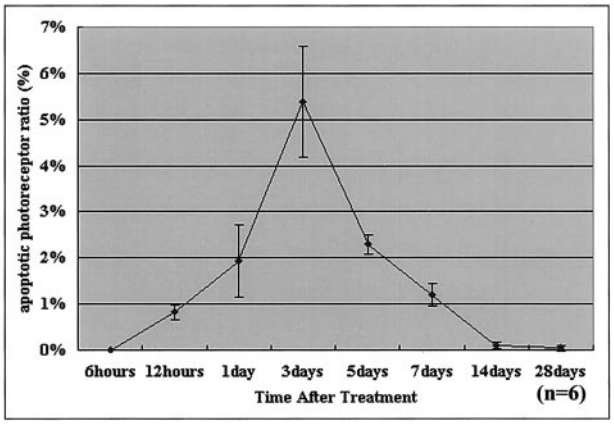

Figure 3. Time course of photoreceptor apoptosis in experimental RD detected by TdT-dUTP TUNEL. The ratio of the number of apoptotic photoreceptors/total photoreceptors increases for 3 days after detachment and then gradually decreases. Apoptotic photoreceptor ratio (%) was evaluated as the ratio of total number of TUNEL-positive photoreceptors/total number of photoreceptors in each section. The sections for each eye specimen were randomly selected and observed by masked observers (six eyes for each time point). The representative results of three independent experiments are presented.

The regular arrangement of mitochondria observed in the control retinas was lost, and AIF staining dispersed from mitochondria to cytosol (Figure 8, A and B). The immunostaining for cytochrome *c* showed that cytochrome *c* was localized in the inner segment of photoreceptors of nondetached retina, whereas it was diffusely dispersed in the cytosol of photoreceptors in the detached (Figure 9). In conclusion, in dying photoreceptor cells both AIF and cytochrome *c* localization altered from mitochondria to an extramitochondrial localization.

Modulation of RD-Induced Photoreceptor Apoptosis by the CD95/CD95 Ligand System and Neurotrophic Factors

In C3H mice, RD induced photoreceptor apoptosis, following a similar time course as that observed in Brown Norway rats, with a maximum of TUNEL-positive cells 3 days after RD. The double staining of AIF and TUNEL confirmed that most of the nuclei from TUNEL-positive photoreceptors also reacted with the AIF-specific antiserum (Figure 10A, arrows). No significant difference was found between these mouse strains as far as the RD-induced AIF- and TUNEL-positive nuclei of photoreceptors (Table 2). The CD95 death receptor is concerned to rapid activation of the caspases. To further exclude the implication of CD95-like receptors in RD-induced apoptosis, RD was induced in rats by subretinal injection of sodium hyaluronate, in the presence or absence of the wide-ranging caspase inhibitor Z-VAD.fmk. No inhibitory effect of Z-VAD.fmk was detected, both at the level of AIF relocalization and at the level of TUNEL positivity. In strict contrast, we found that subretinal application of BDNF significantly reduced the frequency of mitochondrio-nuclear AIF relocalization and TUNEL positivity (Figure 11; day 3, $P < 0.05$). Altogether, these data indicate that the CD95/CD95 ligand system does not control photorecep-

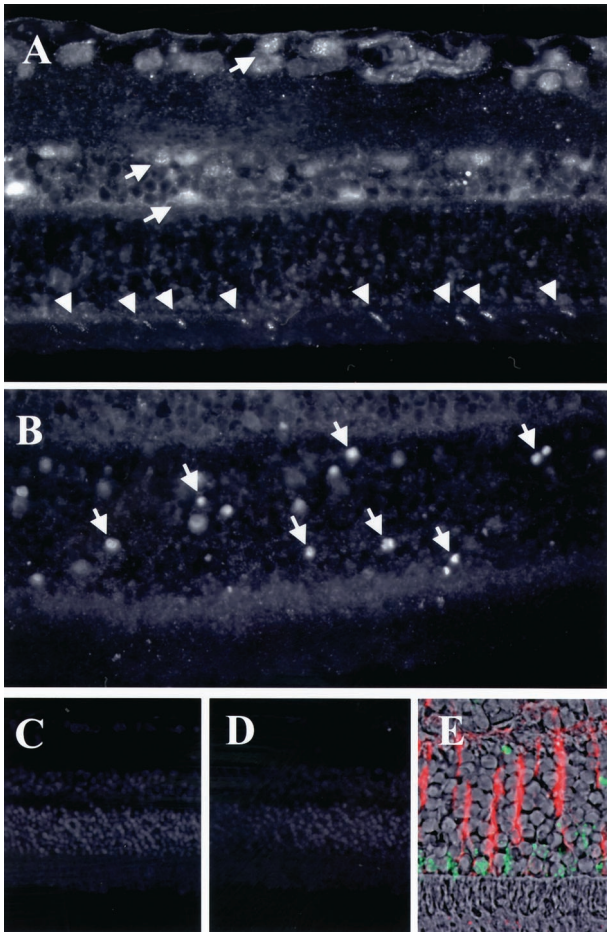

Figure 4. Fluorescent micrographs of AIF immunohistochemistry. The eyes were stained by AIF antiserum and visualized by fluorescent microscopy (see Materials and Methods). **A:** Control retina stained by AIF-antiserum. **B:** Detached retina on day 3 stained by AIF-antiserum. **C:** Control for staining by pre-immune serum. **D:** Control by pre-absorbed antiserum (with 1 μg/μl recombinant AIF). **E:** Double staining for AIF (green) and GFAP (red) on day 1 after RD. Specific AIF-staining is shown in ganglion cells, inner nuclear layer, outer plexiform layer, and photoreceptors (**A**, **arrows**). In normal photoreceptors, AIF staining is localized in inner segment, showing a multilinear pattern (**A**, **arrowheads**). Note the AIF-positive photoreceptor nuclei in the outer nuclear layer of detached retina (**B**, **arrows**). AIF staining decreased from the inner segment and appeared in the nucleus. Original magnifications: ×400 (**A**, **B**, and **E**), ×200 (**C** and **D**).

tor apoptosis, at least in this model. However, local shortage of trophic factors such as BDNF is likely to be involved in RD-induced photoreceptor apoptosis.

Discussion

To our knowledge, this is the first report showing clear evidence of the transition of AIF localization from mitochondria to nucleus in the apoptotic process *in vivo*.

Immunohistochemistry and immunoelectron microscopy revealed AIF to be normally confined to mitochondria, yet to be relocated to the extramitochondrial compartment (cytosol plus nucleus) when nuclear chromatin condensation is occurring. After RD, AIF was found diffusely in the cytosol and in a more patchy manner within areas of condensed chromatin of the nucleus. The double staining for AIF and GFAP showed that most of AIF-positive cells are not Müller cells. Thus, AIF might be involved in the apoptotic process affecting photoreceptor cells.

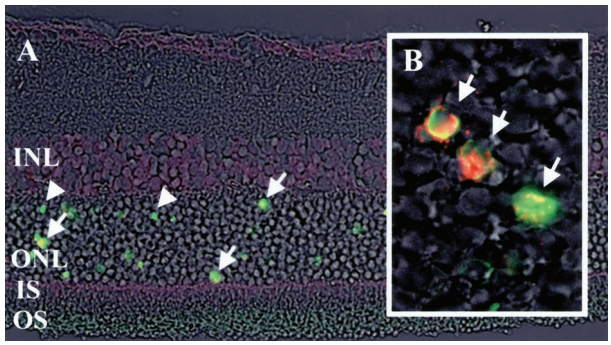

Figure 5. Fluorescent microphotographs of the detached retina, double stained by AIF and TUNEL. Each section was double stained by TUNEL (green) and with AIF antiserum (red), and a micrograph was made using a phase-contrast microscope. **A:** Detached retina (INL, inner nuclear layer; ONL, outer nuclear layer; IS, inner segment; OS, outer segment). Most of AIF-positive photoreceptors (**arrow**) are also TUNEL-positive (**arrowhead**), indicating apoptotic photoreceptors. **B:** High magnification of the outer nuclear layer. Co-localization of TUNEL and AIF staining is shown in detail. Original magnifications: ×400 (**A**), ×1000 (**B**).

Susin and colleagues[9] proved the subcellular translocation of AIF during apoptotic processes by comparing subcellular fractions of mouse liver cells *in vitro*. We also performed both Western blotting of total retinal extracts (with RD) and that of subcellular fraction of the retinal extract for AIF. The results showed that the amounts of total AIF protein remained unchanged for 7 days. We also examined reverse transcriptase-polymerase chain reaction for AIF, and the amount of mRNA for AIF was not significantly changed either. These data might support the translocation of AIF during apoptosis in RD. However the retinal extracts contain not only apoptotic photoreceptors but also photoreceptors with no apoptosis (<5.3% of photoreceptor shows apoptosis at greatest) and many other cellular components, and such other components might mask the intracellular changes of AIF of photoreceptors. Because it is practically impossible to collect the apoptotic photoreceptors exclusively from the detached retina, we could not show the perfect data to prove the translocation of AIF of photoreceptor apoptosis in RD. Therefore, we use the term relocalization of AIF, not the translocation of AIF in this article. From the previous *in vitro* study[9] and the present morphological results, it is conceivable that AIF translocated from the mitochondria to nucleus in apoptosis, however, more evidence is necessary to prove this hypothesis.

Identifying the upstream signal that leads to the mitochondrial and nuclear signs of apoptosis in photoreceptor cells is obviously an important goal. Nonetheless, the

Table 1. Ratio of TUNEL- and AIF-Positive Photoreceptors in Total Photoreceptors in BN Rats

	12 hours	1 day	3 days
TUNEL-positive (%)	0.820%	1.93%	5.38%
AIF-positive (%)	0.724%	1.60%	2.87%

Ten sections for each eye specimen were randomly selected for the evaluation (six eyes for each time point).

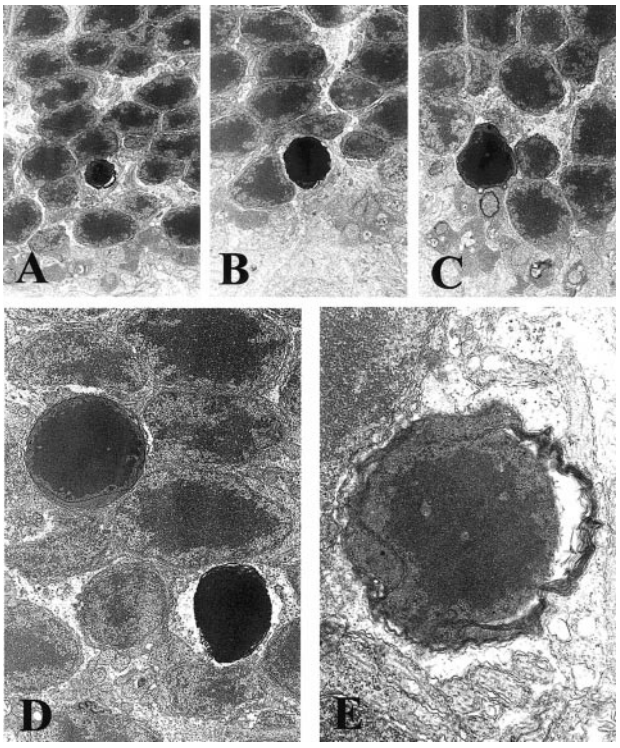

Figure 6. Electron microscopic photographs of the apoptotic photoreceptors. Eyes were fixed in 1% glutaraldehyde and 1% paraformaldehyde in PBS, and embedded in Epon. Major characteristic changes occurring in apoptosis, such as chromatin condensation, alteration of nuclear shape, and cell shrinkage, are seen in the photoreceptors. Original magnification, ×10,000.

mechanisms of photoreceptor apoptosis in experimental RD remains unknown,[18,33–37] and the regulatory mechanism controlling neuronal survival and apoptosis is just the beginning to be defined. In the present study, we found that apoptosis was induced by experimental RD even in CD95/CD95 ligand-deficient animals and that AIF relocalization occurred in these animals in a way undistinguishable from control animals. Photoreceptor apoptosis also developed while downstream caspases were inhibited by the wide-ranging caspase inhibitor, Z-VAD.fmk. Thus, apparently the CD95/CD95 ligand system and downstream caspases are not involved in the photoreceptor apoptosis. In addition, cytochrome *c* immunohistochemistry indicated that photoreceptor apoptosis was mediated by mitochondria via a caspase-independent pathway (Figure 9).

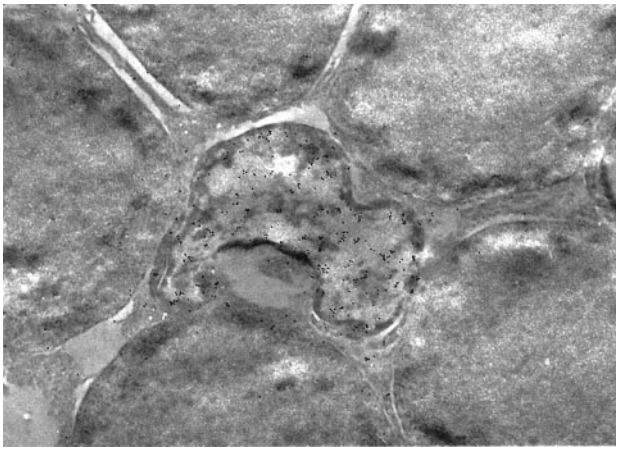

Figure 7. Immunoelectron microscopic photograph of the apoptotic photoreceptors. Apoptotic photoreceptor nuclei shown in Figure 6 are seen as irregularly shaped nuclei in the sections fixed in 1% paraformaldehyde and embedded in LR white. AIF is positive in the cytoplasm and nucleus of apoptotic photoreceptors. The dense staining of AIF on the nucleus is remarkable. Normal photoreceptors are negative in AIF staining. Original magnification, ×10,000.

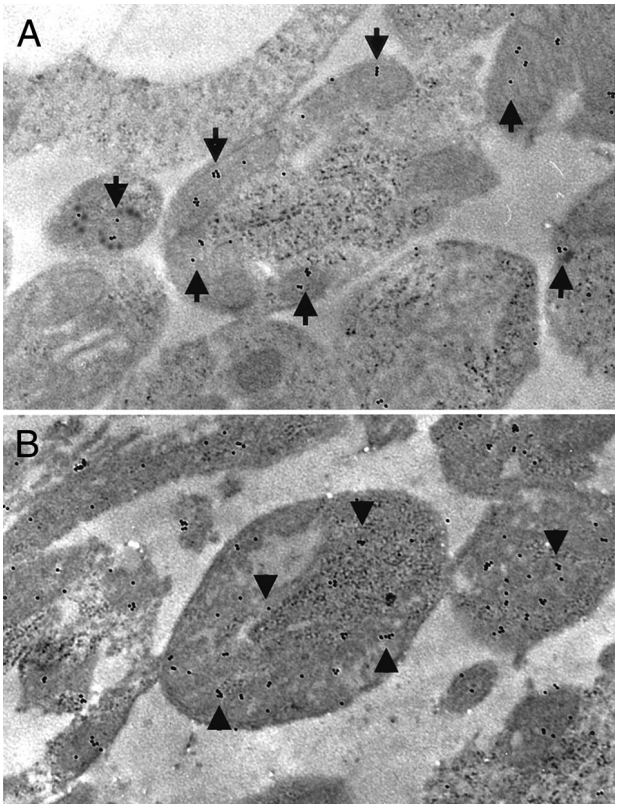

Figure 8. Immunoelectron microscopic photographs of the inner segment of the photoreceptors. **A:** Inner segment of the photoreceptor from the control retina (nondetached). **B:** Inner segment of the photoreceptor from the detached retina (day 3). Mitochondria is placed regularly and the cell structure is well preserved, and AIF staining is confined to mitochondria (**A**, **arrows**). In detached retina, mitochondria degenerates and AIF staining is diffusely distributed in the cytosol of destructive cells (**B**, **arrowheads**). Original magnifications, ×13,000).

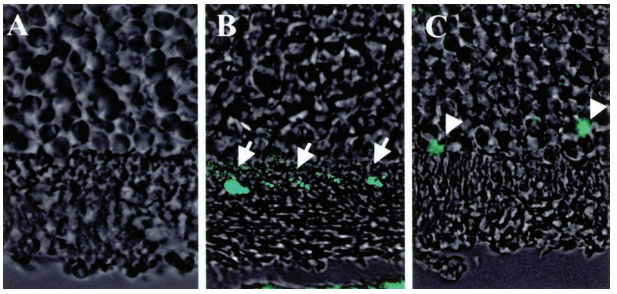

Figure 9. Fluorescent micrographs of cytochrome *c* immunohistochemistry. The eyes were stained by cytochrome *c* antibody and visualized by fluorescent microscopy. **A:** Control for staining without primary antibody. **B:** Control retina stained by cytochrome *c* antibody. Cytochrome *c* is positive in multilinear pattern in the inner segment of the photoreceptor (**arrows**). **C:** Detached retina stained by cytochrome *c* antibody. Cytochrome *c* is positive in the cytosol and nucleus in the outer nuclear layer (**arrowheads**).

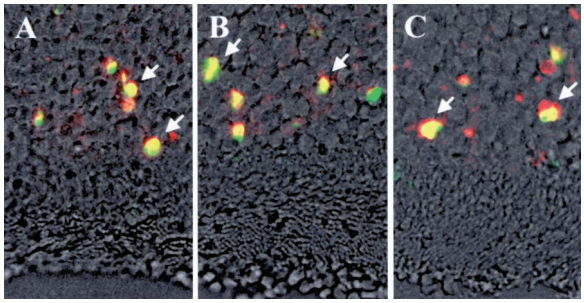

Figure 10. Fluorescent microphotographs of double staining by AIF and TUNEL in CD95/CD-95 ligand gene mutation mice. **A**, C3H, control mice; **B**, C3H-lpr, CD95 gene mutation; **C**, C3H-gld, CD95 ligand gene mutation. AIF immunohistochemistry (red), TUNEL method (green), and phase-contrast microscopy on day 3 after RD. Double-positive photoreceptors (yellow, **arrows**) by TUNEL and AIF are seen in all groups.

Because the inner layers of the retina are supplied by retinal circulation, their nutrient supply is not affected by RD. However, the outer layers, including the photoreceptor layer, are supplied by diffusion from the choriocapillaris, implying that RD greatly compromises their trophic supply and causes nutrients to be diluted in the subretinal space. Mervin and colleagues[33] and Lewis and colleagues[36] already reported that oxygen supplementation (hyperoxia) during detachment reduced photoreceptor death, maintaining the specialized structures of surviving photoreceptors, which is good evidence that photoreceptor death in detached retina is nutrition related. Interestingly, the supplement of a neurotrophic factor such as BDNF into the subretinal space inhibited both the AIF relocalization and the signs of nuclear apoptosis. Neuroprotection by growth factors and neurotrophins such as insulin, insulin-like growth factor-1, nerve growth factor, and BDNF has been studied as a means of regulating neuronal apoptosis.[37] BDNF has been reported to activate extracellular signal-regulated kinase and the phosphatidylinositol-3 kinase (PI3-kinase) pathways. IGF-1-activated phosphoinositide 3-kinase (PI3-K) triggered the activation of the serine-threonine kinase Akt. Akt phosphorylation of the Bcl-2 family member BAD has been demonstrated to promote cell survival.[38,39] The ras mitogen-activated protein kinase signaling pathway also apparently mediates growth-factor-dependent cell survival.[40] However, at the present stage, it remains elusive which among these survival pathways is activated by BDNF in photoreceptor cells.

It remains also an open question whether inhibition of photoreceptor apoptosis will actually restore normal vision. Indeed, the decreased nutrition demands resulting from limited apoptosis might actually reduce total tissue damage. As a result, it will be important to study the long-term effects of apoptosis inhibition by BDNF, overexpression of Bcl-2-like mitochondrioprotective proteins, neutralization of AIF, and similar interventions, not only at the level of cell survival but also in functional terms.

Table 2. Ratio of TUNEL- and AIF-Positive Photoreceptors in Total Photoreceptors in C3H Mice on Day 3

	Wild-type	gld	lpr
TUNEL-positive (%)	5.33%	5.62%	5.11%
AIF-positive (%)	2.92%	3.11%	2.98%

Ten sections for each eye specimen were randomly selected for the evaluation ($n = 6$). No significant difference was found between these mouse strains as far as the RD-induced AIF- and TUNEL-positive nuclei of photoreceptors.

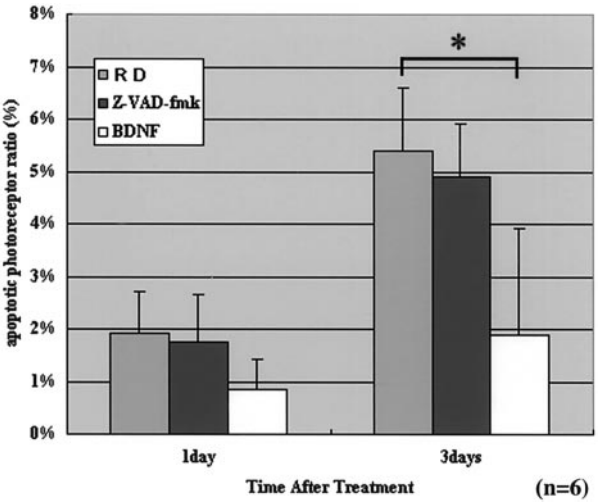

Figure 11. The effect of Z-VAD.fmk and BDNF on photoreceptor apoptosis on days 1 and 3. Ten sections of each eye specimen were randomly selected and observed in a masked manner ($n = 6$). The wide-ranging caspase inhibitor Z-VAD.fmk does not inhibit photoreceptor apoptosis significantly, but the neurotrophic factor BDNF does (on day 3) ($P < 0.05$).

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