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Retinal Cell Death by Light Damage

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Purpose: To determine the relationship between apoptotic photoreceptor cell death and the duration of light exposure.

Methods: Ten-week-old male albino rats (Wistar strain) were dark-adapted for 2 days and then exposed to intense light for 12 hours, and 1, 2, 3, 7, 14, 21, and 28 days. The presence of apoptosis was confirmed by electron microscopy and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method. Differences in the apoptotic processes of the photoreceptor cells in the superior, posterior pole, and inferior portions of the retina were determined.

Results: Photoreceptor cells showed TUNEL-positive staining, whereas the cells in the inner nuclear layer, ganglion cell layer and retinal pigment epithelia exhibited weak positive or negative TUNEL staining. By electron microscopy, photoreceptor cells showed typical apoptotic nuclear changes and formation of apoptotic bodies.

Conclusions: The sensitivity to light damage and style of death differed among retinal cells by location and cell type. **Jpn J Ophthalmol 1999;43:171–179** © 1999 Japanese Ophthalmological Society

Key Words: Apoptosis, electron microscopy, light damage, rat retina, TUNEL method.

Introduction

The term *apoptosis* was proposed by Kerr et al[1] for the active, programmed cell death that is characterized by nuclear and cytoplasmic condensation, and the breaking up of the cell into a number of membrane-bound fragments (apoptotic bodies). The apoptotic bodies are taken up by other cells and rapidly degraded by lysosomal enzymes derived from the ingesting cells.[1] Nuclear chromatin condensation is associated with the enzymatic degradation of the DNA. The formation of a nucleosomal DNA ladder is considered to be one of the hallmarks of apoptosis, but its detection is limited because it requires a large number of cells undergoing apoptosis simultaneously.

Histochemical methods are more sensitive in identifying apoptosis in a small number of cells. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method is one such histochemical method. It is generally considered that the accurate identification of apoptosis requires a combination of morphological criteria obtained by electron microscopy and by the TUNEL method.[2]

Photoreceptor cell death induced by light toxicity is an unusual process. Kuwabara and Gorn[3] have already described this a "quick and mysterious process" in which damaged photoreceptor cells disappear without leaving any trace. Recent studies have suggested that apoptosis is a type of photoreceptor cell death induced by retinal light damage.[4–6] Shahinfar et al[4] exposed albino Lewis rats to green fluorescent light and observed early morphological changes in the photoreceptor cells. They considered that their observation of mitochondrial changes and early DNA digestion was consistent with necrosis. However, in scattered photoreceptor cells, cytoplasmic

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densification and margination of nuclear chromatin combined were present, and the lack of inflammation and the protective effect of cyclohexamide were suggestive of apoptosis.[4]

Büchi and Szczesny[5] exposed rats to light after intravenous injection of rose bengal and observed their retinae by light and electron microscopy. They identified two distinct types of photoreceptor cell death: necrosis and apoptosis. These authors did not conclude that the death of photoreceptor cells by light damage was due to apoptosis or necrosis.

The purpose of our study was threefold: first, to examine photoreceptor cell death by electron microscopic observations and by the TUNEL method. Previous studies have used photoreceptor cell counts and measurements of outer nuclear layer (ONL) thickness to examine photoreceptor cell damage. We have also used ONL thickness/whole retinal thickness ratio to examine the protective effect of ascorbic acid[7] and α-tocopherol[8] against retinal light damage. However, if the ONL includes dying photoreceptor cells undergoing apoptosis, neither cell counts nor ONL thickness can be used directly as indices of cell damage. Our second purpose was to examine the relationship between apoptotic photoreceptor cell loss and the duration of light exposure, and to determine the differences in the apoptotic processes of the photoreceptor cells in the superior, posterior pole and inferior portions of the retina. We analyzed these processes statistically to obtain basic data for the study with our light source.

The mechanism of death from light damage in other retinal cells is also controversial. Büchi and Szczesny[5] reported that apoptosis was observed in the ganglion cell layer (GCL), inner nuclear layer (INL), and retinal pigment epithelium (RPE). Shahinfar et al[4] reported that focal regions of RPE exhibited necrosis; Hafezi et al[6] reported apoptosis in the RPE. We did not observe apoptosis in the GCL, INL, or RPE of rat retina damaged by up to 3 days of light exposure.[9] We suspected that this duration of light exposure may have been too short to induce apoptosis in these cells. Thus, the third purpose of our study was to examine morphologically the cell death caused by long duration of light exposure (up to 4 weeks) in the GCL, INL, and RPE.

Materials and Methods

In this study, we followed the guidelines for animal experimentation of the Hamamatsu University School of Medicine. Ten-week-old male albino rats (Wistar strain) were dark-adapted for 2 days and then exposed to continuous intense light for 12 hours, and 1, 2, 3, 7, 14, 21, or 28 days. As reported previously,[9] the light source consisted of three roentgen film viewers. Each viewer had three 15-W daylight fluorescent lights and a white plastic diffuser. They were set on the top and both lateral sides of a transparent plastic cage containing the rats. The illuminance was 2500 lux at the base of the cage. The luminance was 2000 candela/m^2. The wavelength of the light was 390–630 nm. The room temperature was maintained at 24–27°C by air flow. At least three rats were used for each light exposure group, and an additional three rats served as unexposed controls. Animals had free access to water and commercial diet.

After light exposure, each animal was instantly euthanized with a lethal dose of 1.5–3.0 mL intraperitoneal xylazin. Both eyes were immediately enucleated. One eye of each rat was fixed in 10% formalin overnight and embedded in paraffin. After removal of the anterior segments, the other eye was fixed in 2% glutaraldehyde in phosphate-buffered saline (PBS)

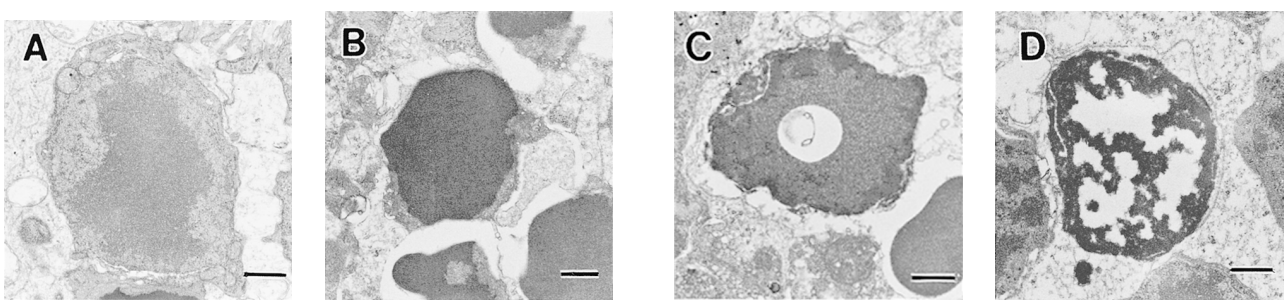

Figure 1. Electron micrographs of photoreceptor cells undergoing apoptotis. (**A**) Normal nuclei group exhibits usual pattern of heterochromatin and euchromatin. (**B**) Pyknotic nuclei group contains over 90% heterochromatin. (**C**), (**D**) Collapsing nuclei group exhibits vacuolization. Vacuolization begins in center of heterochromatin (**C**) and then gradually expands (**D**). Bar: 1 μm.

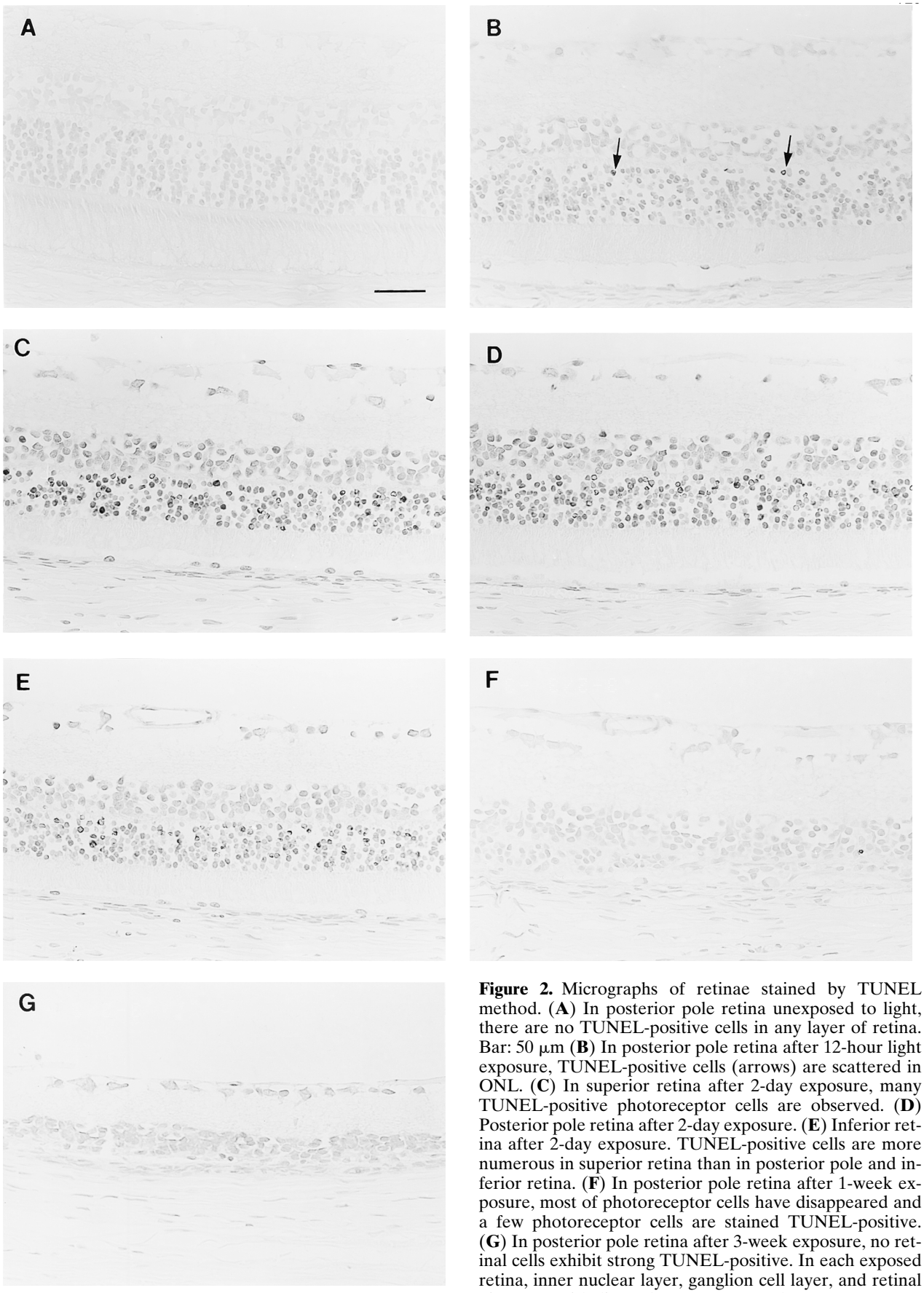

Figure 2. Micrographs of retinae stained by TUNEL method. (**A**) In posterior pole retina unexposed to light, there are no TUNEL-positive cells in any layer of retina. Bar: 50 μm (**B**) In posterior pole retina after 12-hour light exposure, TUNEL-positive cells (arrows) are scattered in ONL. (**C**) In superior retina after 2-day exposure, many TUNEL-positive photoreceptor cells are observed. (**D**) Posterior pole retina after 2-day exposure. (**E**) Inferior retina after 2-day exposure. TUNEL-positive cells are more numerous in superior retina than in posterior pole and inferior retina. (**F**) In posterior pole retina after 1-week exposure, most of photoreceptor cells have disappeared and a few photoreceptor cells are stained TUNEL-positive. (**G**) In posterior pole retina after 3-week exposure, no retinal cells exhibit strong TUNEL-positive. In each exposed retina, inner nuclear layer, ganglion cell layer, and retinal pigment epithelium demonstrate weak or no TUNEL staining.

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(pH 7.4) for 3 hours. The tissues were divided into superior, posterior pole and inferior portions and trimmed to 2 × 2 mm size. They were postfixed with 1% OsO_4 in PBS for 2 hours and embedded in epoxy resin. Sections approximately 70 nm in thickness were cut vertically with a diamond knife and stained with uranyl acetate and Sato's lead solution. They were examined by transmission electron microscopy (JEM 1220; JEOL, Tokyo).

TUNEL Method

The TUNEL method was performed following the protocol of Toné and Tanaka.[10] Paraffin sections 4-mm thick were deparaffinized in xylene and rehydrated through a graded series of alcohol and distilled water (DW). They were treated with proteinase K (10 mg/mL; Boehringer Mannheim, Mannheim, Germany) for 15 minutes at room temperature and washed in DW. Endogenous peroxidase was inactivated by incubating the sections with 2% H_2O_2 for 5 minutes at room temperature and then washed in DW. The sections were incubated with 10-μm biotin-16-dUTP (Boehringer Mannheim), 0.5 U/μL terminal deoxynucleotidyl transferase (TdT; Toyobo, Tokyo) and 20% 5× cacodylate buffer in DW in a moist chamber for 1 hour at 37°C, and washed in PBS. They were treated with peroxidase-conjugated streptoavidine (Zymed) for 30 minutes at room temperature and washed in PBS. Diaminobenzidine was used as a chromogen. Counterstaining was performed with methyl green.

Electron Microscopic Findings and Cell Counts

On electron micrographs at magnification of ×2,000–3,200, photoreceptor cells were classified into three groups: the normal nuclei group (Figure 1A), the pyknotic nuclei group (Figure 1B), and the collapsing nuclei group (Figures 1C, 1D). The numbers of cells classified in the three groups were counted at 1000-μm intervals. Normal nuclei were defined as those with the usual pattern of heterochromatin and euchromatin. Pyknotic nuclei were defined as those containing over 90% heterochromatin. Collapsing nuclei were defined as those with vacuolization in the heterochromatin. The vacuolization began in the center of the heterochromatin (Figure 1C) and then expanded gradually (Figure 1D). To analyze the apoptotic process of photoreceptor cells, we counted the number of cells in the normal nuclei group, in the pyknotic nuclei group, in the collapsing nuclei group, and the number of cells in the control retina other than those of the above three groups and compared the cell counts of these four groups in each retinal area, using the Mann-Whitney *U*-Test.

While examining the ONL, it was our impression that the progress of photoreceptor cell death differed between the inner and outer zones. Therefore, we compared the counts of apoptotic cells (pyknotic nuclei group and collapsing nuclei group) in the inner and outer halves of the ONL after 24-hour light exposrue. Cell counts were performed at 50-μm intervals. Statistical analysis was performed using the paired *t*-test and Mann-Whitney *U*-Test.

The numbers of cells in the INL of the superior, posterior, and inferior retina were also counted at 50-μm intervals. Data were analyzed by Scheffe's test as a post-hoc test after analysis of variance.

Results

TUNEL Method

Figures 2A–G show retinae stained by the TUNEL method after light exposure for variable periods. All the cells in the unexposed retina were negative for TUNEL staining (Figure 2A). After 12-hour exposure, TUNEL-positive cells were scattered in the ONL (Figure 2B). After 2-day exposure, TUNEL-positive photoreceptor cells were more numerous in

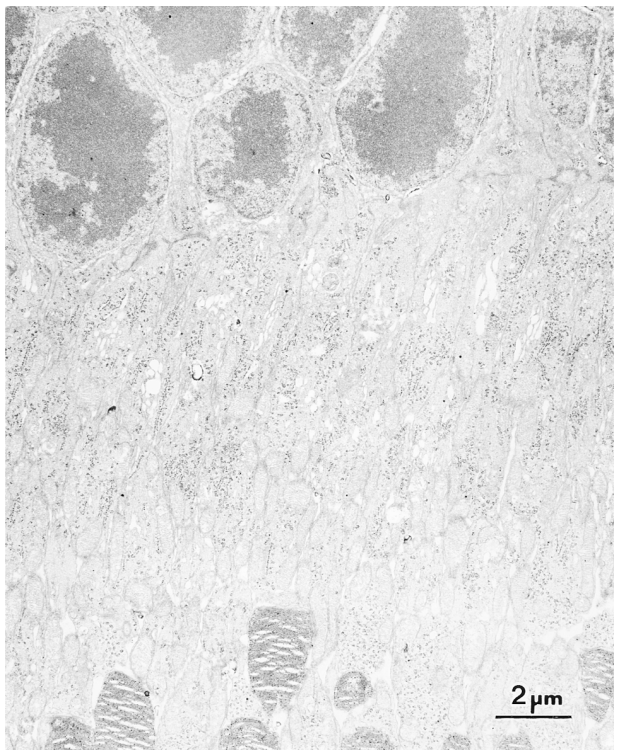

Figure 3. Electron micrograph of inferior control retina show no mitochondrial change in inner segments.

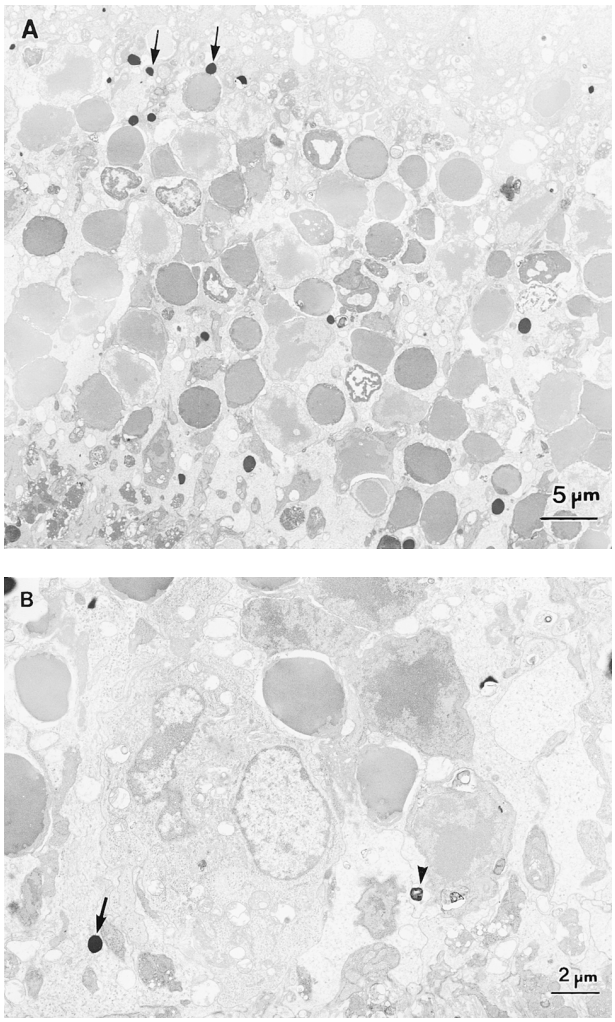

Figure 4. (**A**) Electron micrograph of posterior pole retina after 2-day exposure showing many apoptotic bodies (arrows). (**B**) They are phagocytosed by neighboring Müller cells (arrow) or photoreceptor cells (arrowhead).

the superior retina (Figure 2C) than in the posterior pole (Figure 2D) and the inferior retina (Figure 2E). Up to 3-days exposure, the TUNEL-positive photoreceptor cells increased in number and the total number of photoreceptor cells decreased. Photoreceptor cells were seldom observed in the retinae after 1- or 3-week exposure (Figures 2F, 2G). The cells in the GCL, INL, and RPE exhibited weak or no TUNEL staining in all light-exposed retinae.

Electron Microscopic Findings and Cell Counts

Photoreceptor cells. In unexposed retinae, all the retinal cells were morphologically normal. There were no mitochondrial changes in the inner segments (Figure 3). As the exposure time increased, photoreceptor cells exhibited chromatin densification and subsequent chromatin margination, and decreased in number. Electron-dense granules, presumed to be apoptotic bodies, were often observed in the retinae exposed to light for 2–3 days. They appeared to have been phagocytosed by neighboring Müller cells or photoreceptor cells (Figures 4A, 4B). These electron microscopic findings provided some evidence of apoptosis. However, some mitochondria in inner segments demonstrated swelling and vacuolation after short periods of light exposure (Figure 5). Figures 6A and 6B show the progress of photoreceptor cell death. Nuclear changes in photoreceptor cells began after a 12-hour light exposrue and developed rapidly up to 3-days exposure. Some photoreceptor cells survived 3 weeks in the superior and posterior pole of the retina, and 4 weeks in the inferior retinae. Cell death in the superior retina occurred earlier than in the posterior pole and inferior retina. There was no significant difference among the three areas of control retina in analysis by Mann-Whitney *U*-Test. However, there were statistically significant differences in the progress of apoptosis in the three areas for every light exposrue duration ($P < .001$).

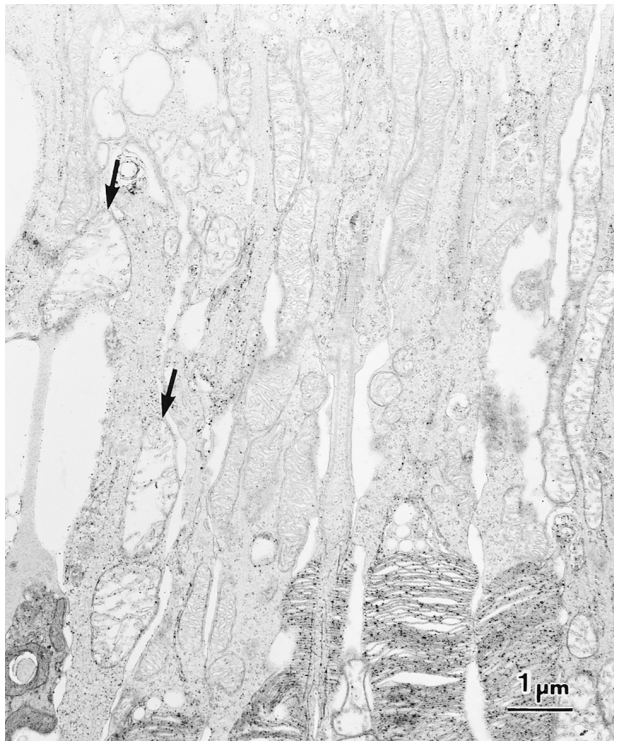

Figure 5. Electron micrograph of inferior retina after 12-hour exposure demonstrating mitochondrial swelling in inner segments (arrows).

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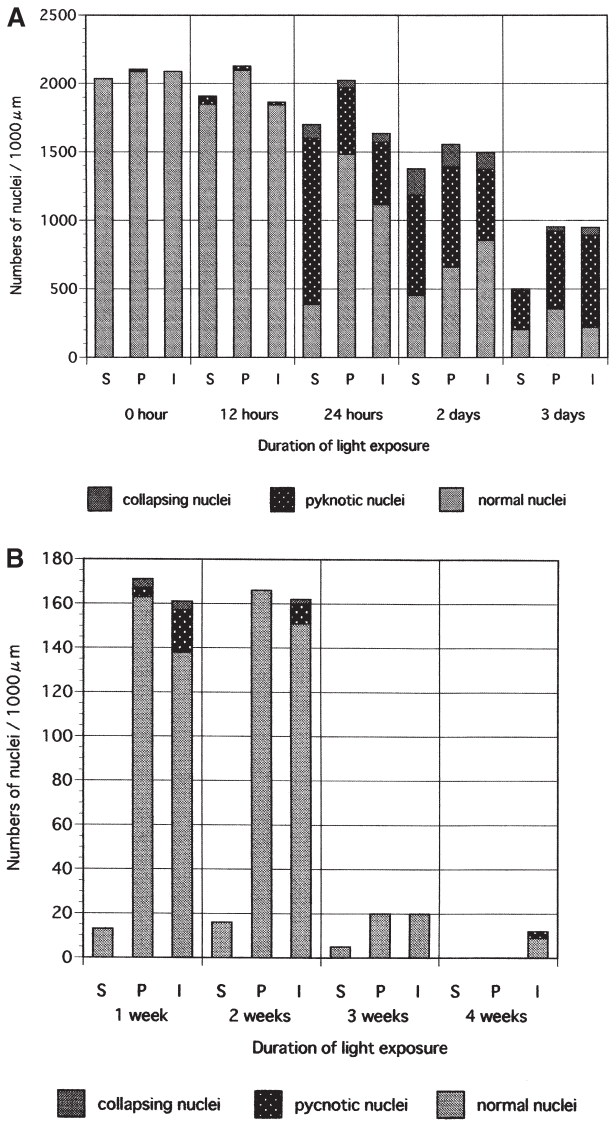

Figure 6. Progress of photoreceptor cell death (**A**) from 12-hour to 3-day exposure, (**B**) from 1- to 4-week exposure). Baseline: 1,000 μm. S: superior retina, P: posterior pole retina, I: inferior retina. Apoptosis of photoreceptor cells was first observed after 12-hour exposure, and increased rapidly until 3-day exposure. Some photoreceptor cells, however, survived 3 weeks in superior and posterior pole retina, and 4 weeks in inferior retina. Cell death occurred earlier in superior retina than in posterior pole and inferior retina. There were significant differences (Mann-Whitney *U*-Test, $P < .001$) in progress of apoptosis in three portions in every light exposure duration except control.

The nuclear changes developed earlier in the inner zone than in the outer zone of the ONL (Figure 7). The mean numbers of apoptotic cells (pyknotic nuclei group and collapsing nuclei group) in the inner and outer halves of the ONL in the retina after

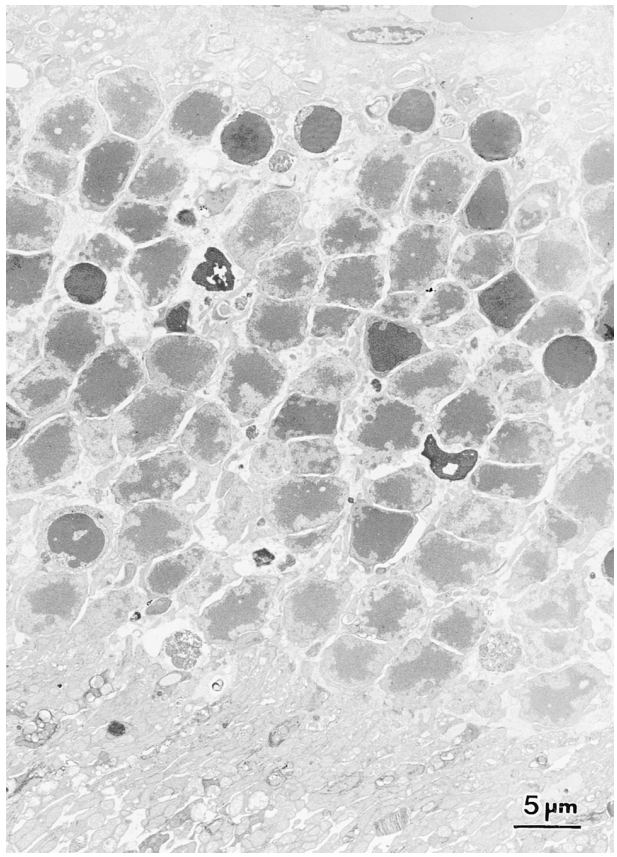

Figure 7. Electron micrograph of inferior retina after 12-hour exposure. Apoptotic nuclei are more numerous in inner half of outer nuclear layer than in outer half.

24-hour light exposure are shown in Table 1. Cell counts were performed every 50 μm along the ONL. In all areas of the retinae, apoptotic photoreceptor cells were more numerous in the inner half of the ONL than in the outer half. There were significant differences between the two halves in the posterior pole and inferior retina ($P < .05$). Severely damaged retinae demonstrated surviving photoreceptor cells with large nuclei that contained multiple small clumps of heterochromatin in the outer zone of the ONL (Figure 8). These nuclear findings suggested the surviving cells were cones.

INL, GCL, and RPE

Cells in the INL decreased in number as exposure time increased (Figure 9). In retinae, after 3- and 4-week exposure, most horizontal and amacrine cells had disappeared. Cells with highly electron-dense nuclei (presumed to be Müller cells) survived in the outer zone of the INL, while those with nuclei with low electron density (presumed to be bipolar cells)

Table 1. Apoptotic Cell Counts in Inner Half and Outer Half of Outer Nuclear Layer

	Superior Retina	Posterior-Pole Retina	Inferior Retina
Inner half	34.6 ± 8.6 (NS)	17.1 ± 5.3*	18.1 ± 2.8*
Outer half	24.6 ± 9.0 (NS)	10.1 ± 2.3*	9.1 ± 1.3*

Note. Mean + SD (bar) of numbers of apoptotic photoreceptor cells/50-μm baseline in inner and outer halves of outer nuclear layer after 24-hour light exposure (n=11).
NS: not significant.
*$P < .05$.

survived in the inner zone (Figure 10). Dying cells in this layer demonstrated lytic destruction: vacuolation of cytoplasm, subsequent disruption of plasma membranes, and karyolysis without condensation of nuclear chromatin or formation of apoptotic bodies (Figure 11). The inner and outer plexiform layers exhibited mitochondrial swelling in the late stage.

Lytic destruction was observed in the GCL (Figure 12). Neither chromatin densification nor apoptotic bodies were observed in the dying cells in the GCL.

Retinal pigment epithelium cells flattened or enlarged to occupy the space formerly occupied by dying photoreceptor cells. They exhibited mitochondrial changes and numerous phagosomes but survived until the late stage. They demonstrated no features of apoptosis.

Discussion

Photoreceptor Cells

Our electron microscopic and TUNEL method findings suggested that photoreceptor cells died by apoptosis. However, there is a problem in considering the process as apoptosis. Can we regard the process, including mitochondrial changes, as apoptosis? In apoptosis, the integrity of the plasma membrane and organelles is usually preserved until the late stage.[1,2] In this study, the membranes of mitochondria in some inner segments were damaged by short-term light exposure. However, the membranes of mitochondria are rich in fatty acids and sensitive to free radicals, suggesting that mitochondria demonstrated vacuolation in the early stage. In this respect, the light-induced apoptosis we observed differed from the other reported forms of apoptosis. The nuclear changes we observed in photoreceptor cells were identical to those of apoptosis. Shahinfar et al[4] regarded photoreceptor cell death induced by light damage as apoptosis followed by necrosis. We consider this cell death to be a modified form of apoptosis.

It has been reported that constant light damages the superior retina earlier than the inferior retina.[11,12] Our statistical analysis demonstrated that apoptosis due to light damage in photoreceptor cells occurred earlier in the superior retina than in the posterior pole and the inferior retinae. As reported by Rapp et al,[12] the average length of rod outer segments in the superior retina is greater than that in the inferior. The rhodopsin concentration in the rod outer segment of the superior retina is greater than

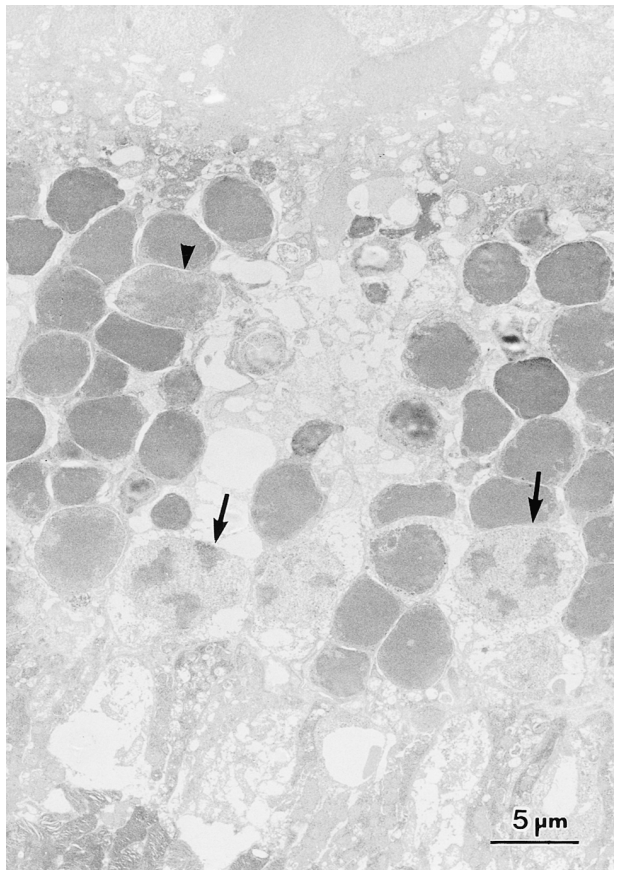

Figure 8. Electron micrograph of superior retina after 3-day exposure. Most photoreceptor cell nuclei demonstrate apoptotic changes. However, some photoreceptor cells survive (arrows). Latter, which are assumed to be cones, are found in outer zone of outer nuclear layer and have large nuclei containing multiple small clumps of heterochromatin. Arrowhead indicates normal rod nucleus.

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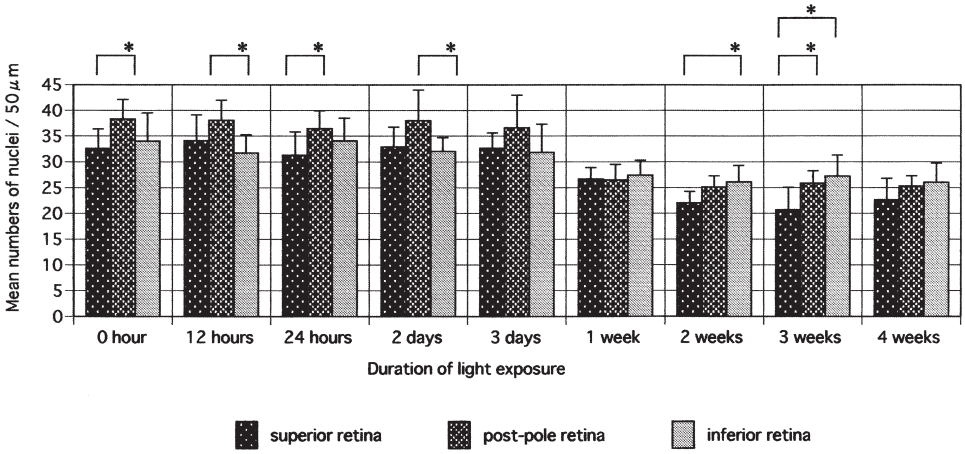

Figure 9. Numbers of cells in inner nuclear layer at intervals of 1,000 μm. Number of cells in this layer decreased as exposure time was lengthened. Asterisks indicate significant difference ($P < .05$) by Scheffe's test as post-hoc test after analysis of variance.

that of the inferior retina. Rhodopsin levels in the superior central retina were found to be 47% greater than in the central inferior retina. Because the action spectrum of light-induced photoreceptor degeneration corresponds to the absorption spectrum of rhodopsin, the superior retina was more susceptible to light damage than the inferior retina.[12]

In rat retina, most photoreceptor cells are thought to be rods. They have small nuclei, usually less than 5.5 μm in diameter with large central clumps. About 1.5% of rat photoreceptor cells are thought to be cones. Their nuclei are larger than 5.5 μm, contain multiple small clumps of heterochromatin, and are located in the outer zone of the ONL.[13] In the present study, most rods died after 2–3 days of exposure, most cones died in 2–3 weeks. Cones appeared to be more resistant to light injury than rods.

Photoreceptor cells died significantly earlier in the inner half than in the outer half of the ONL. This finding has not previously been reported, to the best of our knowledge. The fact that cone nuclei are more numerous in the outer zone of the ONL may partly explain this finding, but cannot completely explain the differences in the apoptotic process between the

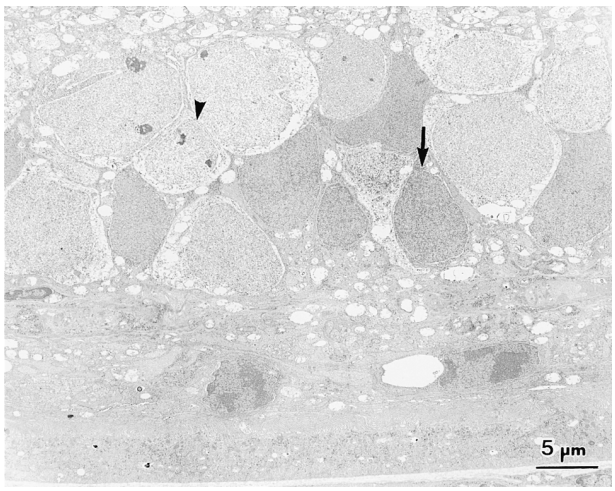

Figure 10. Electron micrograph of superior retina after 3-week exposure. Cells with highly electron-dense nuclei (arrow) survive in outer zone of inner nuclear layer, while those with nuclei with low density (arrowhead) survive in inner zone.

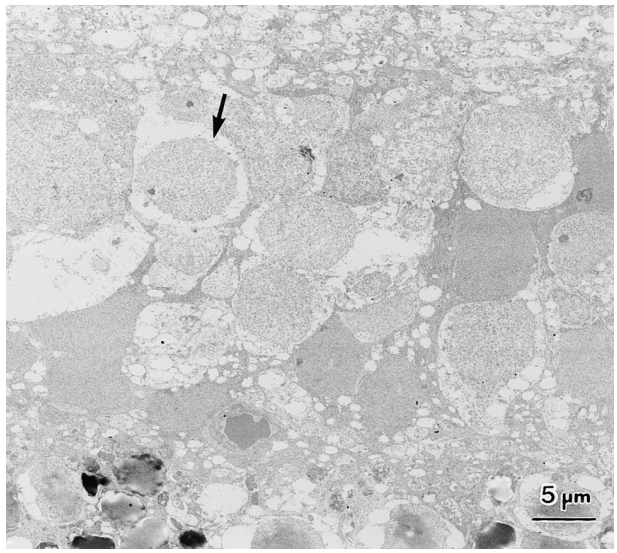

Figure 11. Electron micrograph of superior retina after 2-week exposure. Dying cells in INL demonstrate lytic destruction presumed to be necrosis (arrow). No apoptotic body is observed.

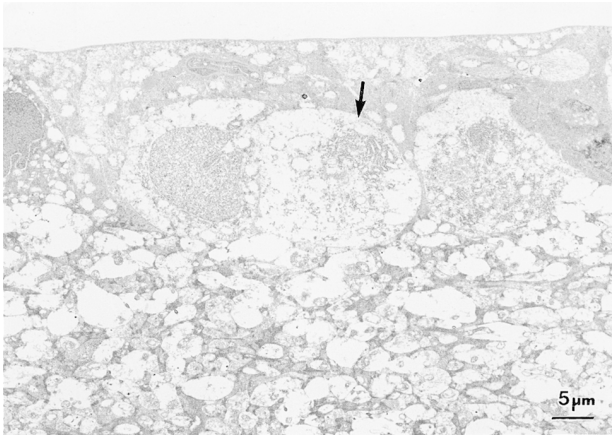

Figure 12. Electron micrograph of posterior pole retina after 1-week light exposure. Ganglion cells are dying because of necrosis (arrow). Inner plexiform layer exhibits mitochondrial swelling in late stage.

two halves. Some other factor may be related to this finding.

INL, GCL, and RPE

Cell numbers in the INL slowly decreased from light damage. They (especially Müller and bipolar cells) were more resistant to light damage than photoreceptor cells. Büchi and Szczesny[2] exposed rats to light after intravenous injection of rose bengal, and reported that apoptosis was observed in the INL, GCL, and RPE. We previously reported that apoptosis was observed in photoreceptor cells, but no morphological change was observed in the inner retinal layer after as long as 72-hours light exposure.[9] In the present study, we prolonged the exposure period and investigated the type of cell death in the INL, GCL, and RPE. Cell death in the INL and GCL occurred by lytic processes without pyknosis or apoptotic bodies, as confirmed by electron microscopy. Some cells exhibited weak TUNEL staining. In general, necrotic cells were also shown to exhibit weak TUNEL staining because they contain fewer DNA fragments than apoptotic cells.[2,10] The deaths of INL and GCL cells appeared to be due to necrosis. Retinal pigment epithelium cells were resistant to light damage and survived until the late stage. They did not demonstrate apoptotic features. However, there was some chance that we overlooked the rapid process of apoptosis, even though we examined nearly 600 electron micrographs. Büchi and Szczesny's[5] report and ours probably resulted from differences between the purposes of the two studies: the purpose of our study was to induce photochemical reaction, and that of Büchi and Szczesny's study was to produce photothrombosis with rose bengal.

Interestingly, the types of death of photoreceptor cells and other retinal cells differed, despite the common origin of these cells. Additionally, it is unclear why cones were more resistant to light damage than rods, and why Müller and bipolar cells were more resistant than the other cells in the INL. We will investigate these problems by examining apoptotis regulating factors and the distribution of free radicals produced by light exposure.

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