Exp. Eye Res. (1997) **64**, 963–970

Light-induced Apoptosis: Differential Timing in the Retina and Pigment Epithelium

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(*Received Seattle 21 November 1996 and accepted in revised form 23 January 1997*)

Apoptosis is a genetically regulated form of cell death. Individual cells show condensed nuclear chromatin and cytoplasm, and biochemical analysis reveals fragmentation of the DNA. Ensuing cellular components, apoptotic bodies, are removed by macrophages or neighboring cells. Genes involved in the regulation of apoptosis as well as stimuli and signal transduction systems, are only beginning to be understood in the retina. Therefore, we developed a new in vivo model system for the investigation of events leading to apoptosis in the retina and the pigment epithelium. We induced apoptosis in retinal photoreceptors and the pigment epithelium of albino rats by exposure to 3000 lux of diffuse, cool white fluorescent light for short time periods of up to 120 minutes. Animals were killed at different time intervals during and after light exposure. The eyes were enucleated and the lower central retina was processed for light- and electron microscopy. DNA fragmentation was analysed in situ by TdT-mediated dUTP nick-end labeling (TUNEL) or by gel electrophoresis of total retinal DNA. We observed that the timing of apoptosis in the photoreceptors and pigment epithelium was remarkably different, the pigment epithelium showing a distinct delay of several hours before the onset of apoptosis. In photoreceptors, apoptosis was induced within 90 minutes of light exposure, with the morphological appearance of apoptosis preceding the fragmentation of DNA. In the pigment epithelium, the morphological appearance of apoptosis and DNA fragmentation were coincident. Different regulative mechanisms may lead to apoptotic cell death in the retinal photoreceptors and pigment epithelium. This in vivo model system will allow measurement of dose-responses, a potential spectral dependence and the molecular background of apoptotic mechanisms in the retina.

Key words: rat retina; apoptosis; gene expression; light damage; retinal degeneration; retinal pigment epithelium.

1. Introduction

There are two main modes of cell death: apoptosis and necrosis. Necrosis includes swelling and lysis of cells with damage of neighboring tissues due to release of lytic enzymes and toxic cellular products. Apoptosis, by contrast, implies a regulated death of individual cells without major effects on the surrounding tissue. Specific morphological and biochemical changes characterize apoptosis, including nuclear chromatin condensation, cytoplasmatic condensation, membrane blebbing and, on the molecular level, internucleosomal fragmentation of nuclear DNA. However, the morphological and biochemical correlates of apoptosis can vary in different tissues. Apoptosis was first discovered in developing tissues and the term programmed cell death (PCD) was used to describe a tightly regulated process in tissue- and organ remodeling (Wyllie, Kerr and Currie, 1980). Apoptosis is also found in a multitude of non-developing tissues and in vitro systems, and multiple stimuli and pathways lead to the induction or inhibition of apoptosis (Reed, 1994; Schwartzman and Cidlowski, 1993; Soares, Curran and Morgan, 1994; Vaux, Haecker and Strasser, 1994).

In the retina, apoptosis is observed during development (Young, 1984), in human retinitis pigmentosa (Li and Milam, 1995) and in animal models of this retinal dystrophy during the period of enhanced cell loss (Adler, 1996; Chang, Hao and Wong, 1993; Lolley, Rong and Craft, 1994; Papermaster and Windle, 1995; Portera-Cailliau et al., 1994). Moreover, recent studies revealed that apoptosis is the mechanism of death in light-damaged photoreceptors. Apoptosis of photoreceptors can be induced by exposure to continuous or intermittent green light for extended time periods or by exposure to diffuse, white light for short intervals (Abler et al., 1996; Remé et al., 1995; Szczesny, Munz and Remé, 1995). Furthermore, we recently showed that light-damaged cells of the retinal pigment epithelium (RPE) also die by apoptosis (Remé et al., 1995). Those light exposure regimens suggest that the apoptotic response is mediated by the visual pigments and phototransduction. On the other hand, a response not directly mediated by rhodopsin bleaching cannot be entirely excluded. In both cases, the question remains whether light-induced apoptosis requires de novo gene expression and/or whether components of the complex apoptotic network are constitutively present in the retina.

In order to approach those questions, a model

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0014–4835/97/060963+08 $25.00/0/ey970288

system was needed for controlled induction of apoptosis in the retina and RPE. This system we have developed consists of in vivo illumination of dark-adapted rats with diffuse, white fluorescent light for short time periods. We have investigated the timing of apoptotic events in retinal photoreceptors and RPE cells. We have found that retinal photoreceptors and cells of the RPE show very different kinetics of cell death. These data will provide the baseline for further studies on gene expression as well as potential mechanisms involved in the induction of apoptosis in retinal photoreceptors versus the RPE.

2. Materials and Methods

Animals

All procedures concerning animals in this study adhered to the ARVO resolution for the care and use of animals in vision research. Male ZUR-SIV albino rats were kept in a regular 12:12 hr light-dark cycle with lights on at 0600 hr and an illuminance level of about 10 lux within the cages. Water and food were provided ad libitum and the room temperature was 22°C.

Light Damage

Rats of 300 g (8–10 weeks of age) were dark adapted for 36 hr and killed or exposed to diffuse, cool, white fluorescent light (Philips TLD-36) of 3000 lux for 2 hr (0800 hr to 1000 hr) and killed at different time points during and after the end of light exposure. Particular care was taken that the rats did not hide their heads so that the eyes were uniformly illuminated. The core temperatures were measured and were found not to increase by more than 1°C during illumination. Groups of rats were killed at 90 and 120 minutes during light exposure. Other groups of rats were killed at the following time points after light exposure: 5 hr, 10 hr, 18 hr, 24 hr, 36 hr and 72 hr with the latter time intervals after exposure spent in darkness.

Morphological Analysis

Rats ($n = 3$ per time point) were decapitated in dim red light and the eyes were rapidly enucleated, placed in 2·5% glutaraldehyde in 0·1 m cacodylate buffer and the anterior half including the lens was removed under a dissecting microscope equipped with a red filter. After 2 hr, the lower central part of the retina was trimmed under a dissecting microscope, fixed overnight, washed and postfixed in osmium tetroxide, dehydrated and embedded in Epon. Semithin and thin sections of the lower central retina were cut, stained with toluidin blue 2% and analysed by light- and electron microscopy (Hitachi 7000).

In situ Nick End Labeling of DNA Strand Breaks

After decapitation of the rats ($n = 3$ per time point), eyes were enucleated and fixed in 2% paraformaldehyde in 0·1 m cacodylate buffer for 2 hr followed by dehydration and paraffin embedding. TdT-mediated dUTP nick-end labeling (TUNEL method) (Gavrieli, Sherman and Ben-Sasson, 1992) was performed with modifications using an 'In situ Cell Death Detection Kit' (Boehringer Mannheim, Germany). DNA strand breaks were labeled with fluorescein and visualized with a FITC filter on a Zeiss Axiophot microscope equipped with a fluorescence illumination system (excitation: 450–490 nm, emission: 510–520 nm).

DNA Fragmentation Analysis

After decapitation of the rats ($n = 2$ per time point), the retinas were gently removed through a slit in the cornea (Winkler and Giblin, 1983) and immediately frozen in liquid nitrogen. Retinas of two animals were pooled. Extraction of retinal DNA and agarose gel electrophoresis were performed as described (Strange et al., 1992). Ten micrograms of total retinal DNA were loaded in each slot and separated on an 1·8% agarose gel. DNA was visualized at 312 nm by staining with ethidium bromide, compared with a 100 bp ladder molecular weight marker (Pharmacia Biotech, Uppsala, Sweden) and photographed using a Polaroid MP-4 system.

3. Results

Timing of Light-induced Apoptosis in the Retina and RPE

After 36 hr of dark adaptation, the retinas appeared morphologically normal [Fig. 1(a)]. After 90 min of light exposure, initial signs of apoptosis were seen in the outer nuclear layer (ONL), consisting of condensed photoreceptor nuclei and few scattered condensed rod inner segments (RIS) [Fig. 1(b)]. Rod outer segments (ROS) showed disruption and vesiculation of the disks. After 2 hr of light exposure, numerous nuclei in the ONL were distinctly condensed and the RIS showed darkened cytoplasm [Fig. 1(c)]. The RPE contained abundant fresh phagosomes but showed no other light related alterations [Fig. 1(c)]. In contrast to the photoreceptors, the first signs of apoptosis in RPE cells were distinctly delayed. Five hours after the end of light exposure, the outer and inner segments of photoreceptors showed distinct signs of deterioration and the ONL contained numerous condensed nuclei. The RPE cells were only slightly swollen with abundant phagosomes in their cytoplasm, but their nuclei displayed discrete initial signs of peripheral chromatin clumping [Fig. 1(d)]. Ten hours after light exposure, the ONL was dramatically altered with dense and shrunken nuclei and the RIS had deterio-

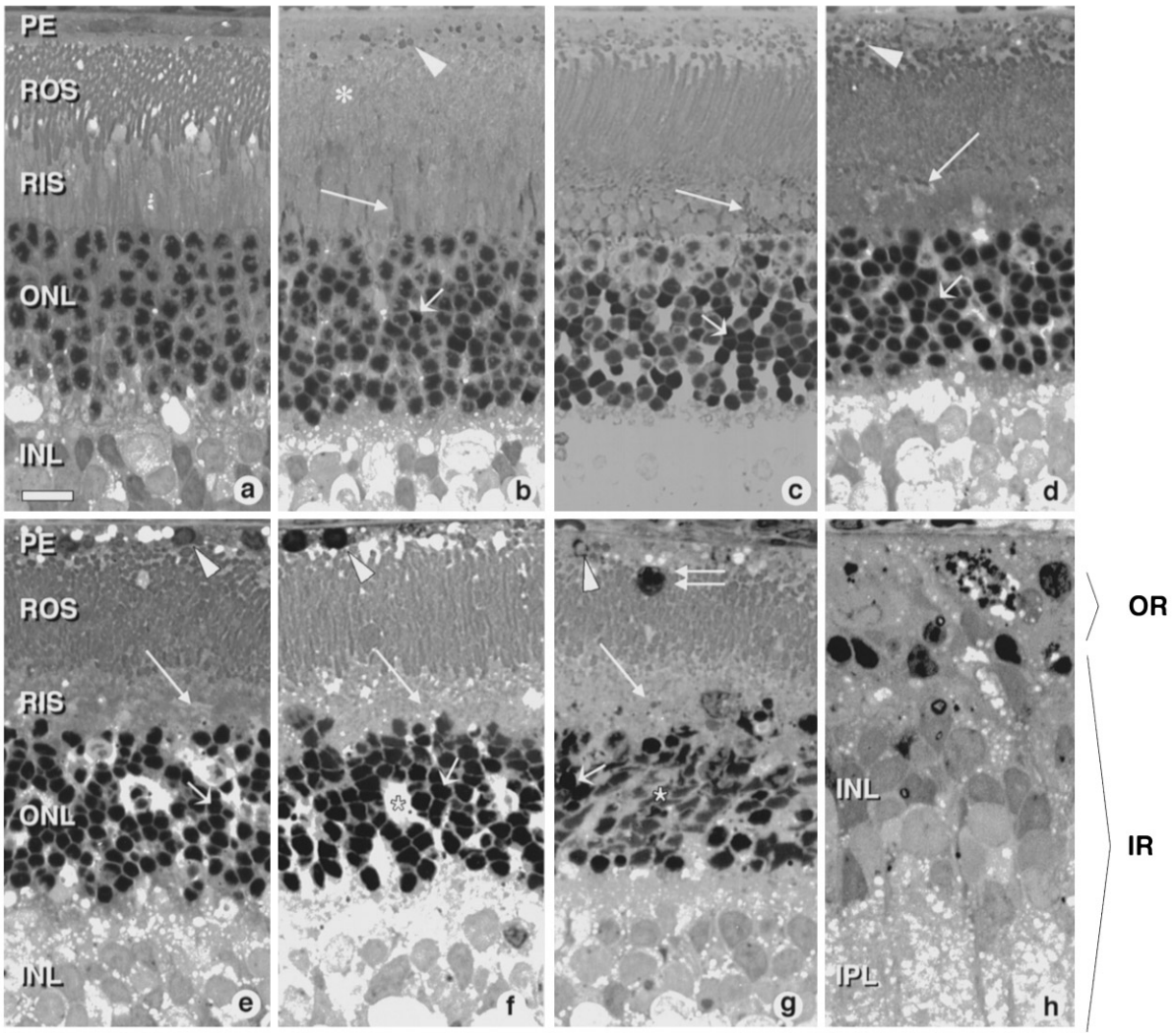

FIG. 1. Light micrographs of apoptotic responses in the lower central part of retinas exposed to 3000 lux and killed at different intervals during and after light exposure. Retina from a dark adapted rat showing regular features shown in (a). After 90 min of exposure, the RPE showed a burst of disk shedding (Δ), ROS revealed disruptions and vesiculations (*), RIS showed scattered condensations of their cytoplasm (long arrow), and few condensed nuclei appeared in the ONL (short arrow) (b). After 2 hr of exposure, RIS revealed distinct condensations of their cytoplasm (long arrow) and the ONL showed numerous condensed nuclei (short arrow) (c). Five hours after the end of light exposure, the RPE revealed large amounts of phagosomes (Δ), ROS were disoriented and RIS showed signs of deterioration and heavily condensed cytoplasmic regions (long arrow), most nuclei in the ONL were distinctly condensed (short arrow) as shown in (d). Ten hours after the end of light exposure, the RPE showed signs of apoptosis with peripheral chromatin clumping (Δ). ROS showed disruptions and RIS (long arrow) were deteriorated, nuclei in the ONL were shrunken and condensed (short arrow) (e). Twenty-four hours after the end of light exposure, the RPE showed distinct apoptotic nuclei which were heavily condensed (Δ), RIS and ROS were disintegrating (long arrow), nuclei in the ONL were shrunken and pycnotic (short arrow), spaces (*) may indicate cell loss (f). Thirty-six hours after the end of light exposure, RPE cell nuclei were disappearing (Δ), ROS and RIS were disintegrated (long arrow), the ONL presented mostly fragmented nuclei (*) with few condensed nuclei remaining (short arrow). Macrophages (double arrow) were distinct (g). Seventy-two hours after the end of light exposure, the outer Retina, OR (RPE, ROS, RIS and ONL) was practically removed, the Inner Retina (INL, IPL) was well preserved as shown in (h). RPE: retinal pigment epithelium, ROS: rod outer segments, RIS: rod inner segments, ONL: outer nuclear layer, INL: inner nuclear layer. OR: outer retina. IR: inner retina. Scale bar = 10 μm. Magnification factor: 688 ×.

rated. The RPE showed distinctly condensed nuclear chromatin and cystoid swelling of the cytoplasm [Fig. 1(e)]. These changes progressed at 24, 36 and 72 hr after light exposure, with cellular breakdown and accumulation of apoptotic bodies within the ONL, which contained only a few remaining condensed nuclei and showed deterioration of the ROS and RIS. The RPE cells were flattened and occasionally detached from their basement membrane, the cytoplasm was mostly condensed, and the nuclei were highly condensed [Figs. 1(f–h)]. Macrophages were seen from 24 hr onwards throughout the outer retina (choroid—RPE, ROS, RIS and ONL) [Fig. 1(g)]. The macrophages increased in number from about 2–3% per unit area at 24 hr to about 4–5% per unit area at 36 hr. Seventy-two hours after light exposure, the ONL and RPE contained only a few remaining pycnotic nuclei and abundant cellular remnants in the region of the

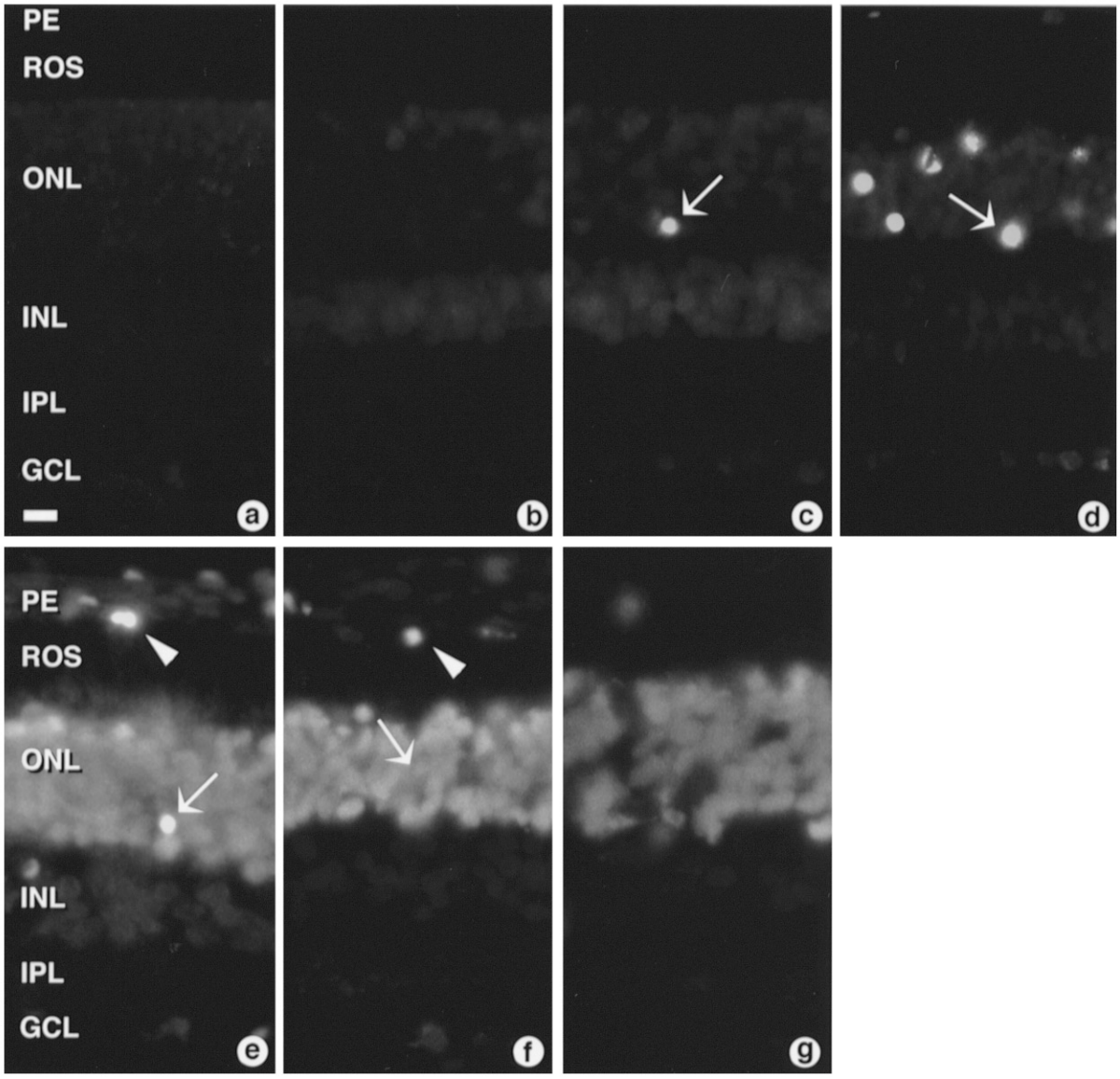

FIG. 2. Detection of DNA strand breaks in photoreceptor nuclei and RPE in light microscopic sections by in situ nick end-labeling (TUNEL). The lower central part of retinas from rats that were dark adapted (a) or exposed to light for 90 min. (b) showed no signs of DNA fragmentation. After 2 hr of light exposure, only very few nuclei in the ONL (arrow) were stained, the majority of nuclei being negative as shown in (c). TUNEL labeling became positive 5 hr after the end of light exposure in the ONL (arrow). In contrast, RPE cells showed no labeling yet (d). Ten hours after the end of light exposure, the RPE revealed positive labeling (Δ) and the ONL showed labeling in almost every nucleus (arrow) (e). DNA fragmentation in the ONL and RPE remained positive after 24 hr (f) and up to 36 hr in the ONL (g) after the end of light exposure. RPE: retinal pigment epithelium, ROS: rod outer segments, RIS: rod inner segments, ONL: outer nuclear layer, INL: inner nuclear layer. Scale bar = 10 μm. Magnification factor: 475 ×.

former ONL [Fig. 1(h)]. A few swollen cells with washed out appearance appeared in the ONL from 12 hr onwards.

DNA Fragmentation in the Retina and RPE

Apoptosis is accompanied by a non-random DNA degradation into oligonucleosomal fragments. DNA fragmentation was measured in situ by a terminal transferase reaction (TUNEL staining). In this assay free DNA ends generated during apoptosis are labeled in histological sections. After 36 hr of dark adaptation, the retinas showed no staining of DNA strand breaks [Fig. 2(a)]. After 90 min of light exposure, no staining of DNA strand breaks was detectable [Fig. 2(b)], whereas initial signs of apoptosis were seen by light microscopy [Fig. 1(b)]. After 2 hr of light exposure, TUNEL staining was still essentially negative [Fig. 2(c)], but light microscopy revealed distinct morphological signs of apoptosis [Fig. 1(c)]. Five hours after exposure there was clear labeling of nuclei in the ONL containing fragmented DNA [Fig. 2(d)], which progressed at 10 hr [Fig. 2(e)]. Twenty-four and 36 hr after light exposure [Fig. 2(f), (g)], the ONL was diffusely labeled. The RPE showed some staining after

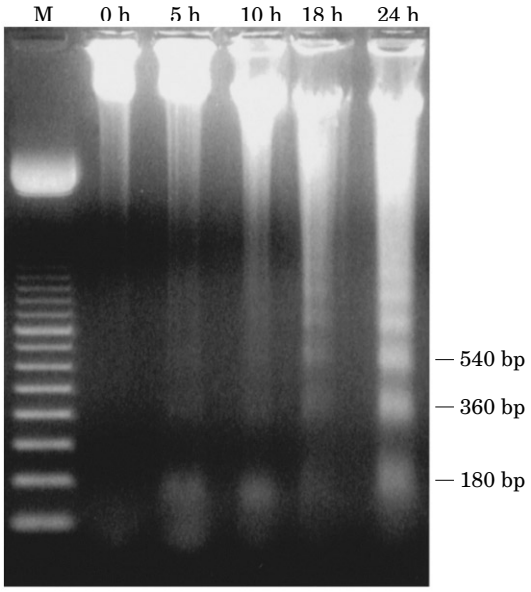

FIG. 3. Analysis of the fragmentation of total retinal DNA by agarose gel electrophoresis. Right after the end of light exposure, no signs of DNA fragmentation were seen (0 hr). Beginning at 5 hr after light exposure, a DNA 'ladder' became increasingly distinct (10 hr, 18 hr, 24 hr).

5 hr and clear staining after 10 hr [Fig. 2(e)], which declined after 36 hr [Fig. 2(g)].

The induction of DNA fragmentation was corroborated by means of extraction of total retinal DNA and analysis by agarose gel electrophoresis. An internucleosomal fragmentation of DNA into breaks of 180 bp and multiples, indicative of apoptotic cell death, was apparent from 5 hr onward (Fig. 3). This DNA ladder became more evident after 10, 18 and 24 hr, correlating with the increasing number of apoptotic nuclei showing DNA fragmentation.

Ultrastructure of Apoptotic Stages in the Retina and RPE

After 36 hr of dark adaptation, photoreceptor [Fig. 4(a)] and RPE [Fig. 4(e)] nuclei were morphologically normal. Early chromatin changes in the ONL appeared as moderate condensation, which progressed towards densely shrunken pycnotic nuclei [Fig. 4(b)]. Chromatin condensation progressed from the center to the periphery of the photoreceptor nucleus. Endstage nuclei were often rounded [Fig. 4(c)] before breaking up into apoptotic bodies [Fig. 4(d)]. Early alterations of the photoreceptor chromatin were visible at 30 and 90 min of light exposure (data not shown), whereas distinct chromatin changes predominated from 2 hr onward [Fig. 4(b)]. In contrast, in the RPE, initial peripheral chromatin clumping was seen only after 5 hr [Fig. 4(f)], progressing from the periphery to the center of the nucleus. Distinct changes were obvious 10 hr after light exposure, whereas after 24 hr, nuclei were shrunken with highly condensed chromatin [Fig. 4(g)]. The cytoplasm of RPE cells showed vesiculation and condensation from 10 hr onward [Fig. 4(f)].

4. Discussion

Our data revealed that apoptosis in photoreceptors was induced by light exposure within 90 min, whereas the RPE showed a delay of 5 hr. Furthermore, it appeared that there was a simultaneous onset of light-induced apoptosis in the retina as well as in the RPE. Positive TUNEL labeling in photoreceptors and nucleosomal laddering of isolated retinal DNA were seen much later than the morphological appearance of apoptosis. In the RPE, by contrast, the TUNEL labeling and morphologic changes were coincident. An increasing number of macrophages was observed from 24 hr onward in the outer retina. The pattern of chromatin condensation was not the same in photoreceptor and RPE cell nuclei. In photoreceptors, condensation of nuclear chromatin started in the center and progressed toward the periphery, whereas RPE cell nuclei showed initial peripheral chromatin clumping which later progressed toward the center of the nucleus.

Regulative Mechanisms and Mediators of Apoptosis

The regulation and potential mediators of apoptosis are not yet known for the retina. Pathways activated by oxidative stress have been described in other cell systems (Buttke and Sandstrom, 1994; Ratan, Murphy and Baraban, 1994; Sandstrom et al., 1994). Oxidative stress may induce de novo gene expression or activate the constitutive cellular components of apoptosis. For light-induced apoptosis, oxidative stress appears to be a plausible mechanism because conditions of light damage are well known to generate photochemical reaction products such as singlet oxygen and various free radicals (Dillon, 1991). In addition, work in our laboratory has revealed a light-evoked release of polyunsaturated fatty acids in the retina (Jung and Remé, 1994; Reinboth and Remé, 1995) and their enzymatic peroxidation, resulting in inflammatory mediators and their intermediates (Reinboth et al., 1995), which can also induce apoptosis (Sandstrom et al., 1994). Indeed, preliminary work in our laboratory demonstrated that arachidonic acid, 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and light exposure can elicit an apoptotic response in the retina in vitro (Reinboth et al., 1996). There may exist a certain analogy to the skin where mechanisms of induction include an immediate apoptotic response evoked by UVA1 through direct membrane damage and a delayed one mediated by UVB and UVC via lesions to DNA and insufficient repair mechanisms (Godar and Lucas, 1995). De novo gene expression may comprise immediate early genes which have been shown to be involved in the regulation of apoptosis and rapidly respond to a variety of exogenous stimuli (Feng et al., 1995; Marti et al., 1994; Morgan and Curran, 1995).

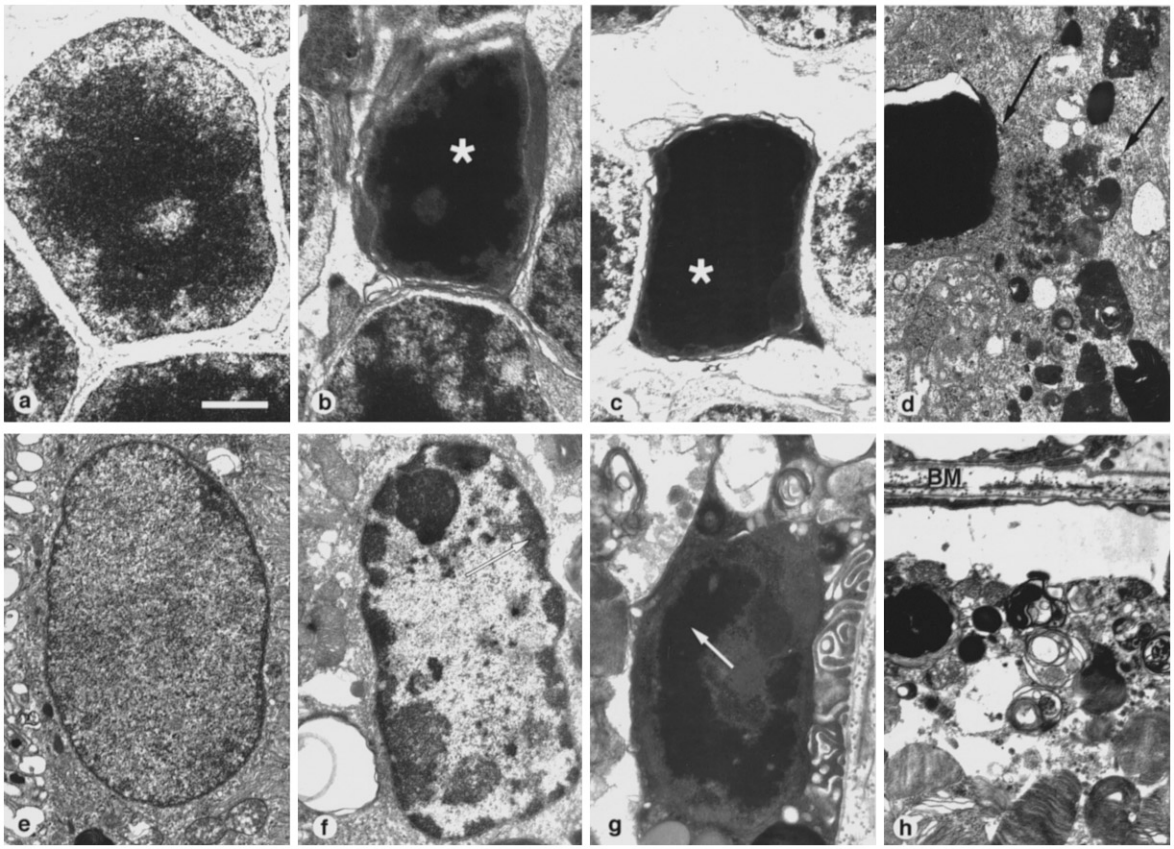

FIG. 4. Electron micrographs of different stages of apoptosis in nuclei of photoreceptors and RPE cells of the lower central retina. **Photoreceptors**: retina from a dark adapted rat. Photoreceptor nuclei showed regular morphology without chromatin alterations as shown in (a). After light exposure to 3000 lux for 2 hr, numerous nuclei showed central chromatin clumping (*) leaving lighter margins (b). Ten hours after light exposure most nuclei were heavily condensed and shrunken (*) as shown in (c). Twenty-four hours after the end of light exposure, apoptotic bodies (arrows) of varying sizes were observed in the Müller cell cytoplasm (d). **Pigment epithelium**: retina from a dark adapted rat. The RPE nucleus showed regular morphology without chromatin alterations (e). Five hours after the end of light exposure. Nuclear chromatin showed initial peripheral clumping (arrow) (f). Twenty-four hour after the end of light exposure. The RPE nucleus was heavily condensed and shrunken, peripheral chromatin was still apparent (arrow) (g). RPE cell showing condensed cytoplasm with deteriorating organelles and dark inclusion bodies as shown in (h). RPE: retinal pigment epithelium. BM: Bruch's membrane. Magnification factor: 5000 ×.

Duration of Apoptotic Stages, Structure-function Discrepancy

The morphological features of apoptosis in photoreceptors were found hours before the biochemical markers were positive. Conceivably, endonucleases were activated at later stages, leaving the question of which molecular events underlie the observed morphological changes of apoptosis. Alternatively, the apoptotic DNA breaks may proceed in several steps with large fragments in the kilobase range occurring first; a similar phenomenon is observed in the rat retina after lead exposure (Poblenz et al., 1995) and in several epithelial cell types (Oberhammer et al., 1993).

Delayed Apoptosis in the RPE

It is noteworthy that apoptosis in the RPE occurred distinctly later than that in photoreceptors. Three possible explanations for the induction of apoptosis in RPE cells can be envisaged. First, it has been well described that the breakdown of cell–cell and cell–matrix interactions triggers apoptosis in several cell types (Frisch and Francis, 1994; Meredith, 1993). This may also account for apoptosis in cells of the RPE, which are structurally and functionally connected to retinal photoreceptors. Once the photoreceptors have died, the cell-cell signaling is lost, an event that may subsequently trigger apoptosis in the RPE. Second, genes regulating apoptosis in the RPE are unknown, but may include those responsive to cytokines. Cytokine release has been observed in the RPE under various conditions (Jaffe et al., 1995; Shima et al., 1995) and cytokines can activate genes regulating apoptosis in other tissues (Tamura et al., 1995). Third, confirming our early observations (Bush, Malnoe and Remé, 1991; Remé et al., 1986), light exposure evoked a major burst of disk shedding, as demonstrated for example in Fig. 1(b). This dramatic and unscheduled accumulation of phagosomes may also release signals to induce cellular responses leading to RPE apoptosis.

Dose-response of Light-induced Apoptosis

Changes observed in our model reflect a dose-response to illuminance levels. Vesiculation and disruption of ROS are usually seen at illuminance levels of 400–800 lux for 1–2 hr; these changes obviously are confined to the ROS (Bush et al., 1991). At 1000 lux for 2 hr, changes then include the entire photoreceptor cell (Szczesny et al., 1995). At this time point, apoptosis was seen in the lower central retina. At illuminances of 3000 lux for 2 hr, however, the entire central retina showed photoreceptor and RPE apoptosis. At later time points from 24 hr onward, macrophage infiltration and an accumulation of cellular debris and apoptotic bodies became more prominent, followed by scar formation from 72 hr. It remains to be seen whether there is an upper limit of illuminance at which apoptotic cell death is replaced by cellular necrosis.

In our model system we observe a fairly synchronized onset and progression of apoptotic cell death. Therefore, pycnotic nuclei and deteriorating RIS and ROS accumulate and may resemble cellular debris resulting from necrosis rather than condensed cellular fragments usually described in apoptosis. However, the molecular analysis of our material clearly indicates apoptosis. The massive apoptotic response after light exposure stands in contrast to the scattered apoptotic cells observed in other experimental models, for example in the rd- and rds-mice or transgenic mice with the Q344ter rhodopsin mutation (Portera-Cailliau et al., 1994).

Cellular fragments and apoptotic bodies were found to remain for longer time periods than usually reported for apoptosis. Again this may be due to the large amounts of cellular remnants that cannot be removed as quickly as single apoptotic cells. Quantification of macrophages in earlier experiments from our laboratory revealed an increase from about 2% at 24 hr after light exposure to about 8% at 48 hr post exposure. Thus, there is a distinct time lag between massive apoptotic cell death and its removal. It is noteworthy that in our experiments, we do not observe invading microglia cells as described in other retinal degenerations (Thanos, 1992), and relatively little phagocytic activity of Müller cells.

In conclusion, our non-invasive in vivo model system of threshold light damage induces a synchronous onset of apoptosis and thus provides baseline values for the timing of apoptosis in the retina and RPE. Therefore, the model provides a useful tool for the molecular analysis of apoptosis in the retina and RPE. With baseline studies in hand, candidate genes for the regulation of apoptosis can be elucidated. Recent studies have shown that degenerating photoreceptor cells in human retinas with retinitis pigmentosa (Li and Milam, 1995) and in animal models of this dystrophy (Adler, 1996; Chang et al., 1993; Portera-Cailliau et al., 1994) die by apoptosis. A better understanding of apoptotic mechanisms in the retina may therefore open alleys for future therapeutic interventions in humans to prevent light-induced retinal injury and postpone the time course of cell death in retinal dystrophies.

Acknowledgements

We thank C. Imsand and L. Vogel for skilled technical assistance. Supported in part by grants from the Swiss National Science Foundation, Nr. 31-40791.94, Sandoz Foundation, Basel, Bruppacher Foundation, Zürich, Switzerland and by Ian and Caroline Leaf and family.

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