Apoptosis Leads to Photoreceptor Degeneration in Inherited Retinal Dystrophy of RCS Rats

Mark O. M. Tso,* Chang Zhang,* Andrew S. Abler,* Cheng-Jong Chang,* Fulton Wong,† Guo-Qing Chang,† and Tim T. Lam*.

Purpose. To determine the pathogenetic mechanism of photoreceptor cell degeneration in the inherited retinal dystrophy of Royal College of Surgeons (RCS) rats.

Methods. The dystrophic retinas of the pink-eyed RCS (RCS-rdy-p) rats were examined for DNA fragmentation by agarose gel electrophoresis of retinal DNA and by TdT-mediated biotin-dUTP nick-end labeling (TUNEL) in paraffin sections. Rats ranging in age from 3 to 60 days were examined.

Results. Agarose gel electrophoresis of retinal DNA isolated from animals 25, 30, 35, and 40 days old showed a ladder pattern of degradation with bands corresponding to multiples of 180 to 200 base pair subunits. TUNEL study showed increasing labeling of photoreceptor cells with progression of the retinal dystrophy of the RCS rats.

Conclusions. Apoptosis is the dominant mechanism of photoreceptor degeneration in the RCS rat, which has a genetic defect in the phagocytic activity of retinal pigment epithelium. The onset of the degeneration appeared to vary between rod cells in the different regions of the eye. Invest Ophthalmol Vis Sci. 1994;35:2693–2699.

The inherited retinal dystrophy of Royal College of Surgeons (RCS) rats is an established animal model for the study of photoreceptor degeneration. 1–5 The primary pathogenetic locus causing the retinal dystrophy is a mutant gene expressed in the retinal pigment epithelium (RPE). The RPE fails to phagocytose the outer segment disks of photoreceptors, which are being continuously shed into the subretinal space, and photoreceptor degeneration follows. Recently, this animal model excited further interest when the photoreceptor cells in the dystrophic retina could be rescued in experimental chimeras, 6 by transplantation of RPE cells from normal rats, 6 or by intravitreal injection of basic fibroblast growth factor. 7 However, the mechanism of photoreceptor cell death has not been determined. An understanding of the mechanisms of photoreceptor cell death in RCS rats should provide additional research avenues for photoreceptor rescue.

Apoptosis, also termed programmed cell death by some investigators, is a specific form of cell demise initiated by an endogenous cellular process that brings about the death of the affected cells. 8–11 This type of signal-induced cell death is believed to be triggered by a gene or by sets of genes and is commonly seen in embryogenesis and morphogenesis to regulate the size and shape of organs by deletion of selective cells. Physiological programmed cell death is seen in the normal turnover of cells in the intestinal epithelium, hormonal-mediated regression of uterine epithelium, prostate epithelium, adrenal gland, regression of lactating mammalian gland, atresia of ovarian follicles, deletion of certain lymphocyte clones, and others. This process is best characterized biochemically by double-stranded DNA degradation in which the genome is cleaved at the internucleosomal sites, producing a ladder pattern of fragments that vary by multiples of 180 to 200 base pairs when resolved by agarose gel electrophoresis.
gel electrophoresis. The endonuclease, which brings about the DNA degradation, appears to be calcium- and magnesium-dependent and may be inhibited by zinc ions. This process appears to require de novo protein synthesis and may be triggered by a rise in intracellular calcium level. Morphologically, apoptotic cells characteristically exhibit condensation of chromatin at the nuclear periphery, nuclear disintegration, reduction in nuclear size with formation of apoptotic bodies, and progressive degeneration of the residual nuclear and cytoplasmic structures.

More recently, Gavrieli described in situ visualization of nuclear DNA fragmentation in conventional histologic sections of small and large intestines, epidermis, lymphoid tissues, ovaries, and other organs with DNA nick-end labeling with biotinylated dUTP, after incubation with a terminal deoxynucleotidyltransferase (TUNEL method). These investigators noted that only nuclei located at positions where programmed cell death was expected were labeled, and they provided a novel method to detect nuclear DNA fragmentation at the single cell level.

This article describes findings from a study of agarose gel electrophoresis of retinal DNA and TUNEL labeling of the dystrophic RCS photoreceptor cells and suggests that massive programmed cell death is the dominant mechanism of photoreceptor degeneration in the RCS rat retina.

MATERIALS AND METHODS

Animals

Pink-eyed RCS (RCS-rdy-p) dystrophic rats were obtained from the National Institutes of Health and were bred and reared in our vivarium in 12-hour cycles of light (five footcandles) and dark. Six normal adult Lewis rats aged 35 to 55 days were used as a control. The care and maintenance of the rats conformed to the ARVO Resolution on the Use of Animals in Research. The animals were given a fatal overdose of sodium pentobarbital, their eyes were enucleated, and their retinas were obtained for DNA fragmentation analysis or TdT-mediated biotin-dUTP nick-end labeling (TUNEL method). These investigators noted that only nuclei located at positions where programmed cell death was expected were labeled, and they provided a novel method to detect nuclear DNA fragmentation at the single cell level.

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In addition, at least two retinas from RCS rats aged 3 (2), 10 (2), 20 (4), 25 (2), 30 (2), 35 (2), 45 (2), and 60 (2) days were fixed in 2% paraformaldehyde/1% glutaraldehyde, embedded in epon, and processed for detailed examination by light and electron microscopy by standard methods.

RESULTS

The agarose gel electrophoresis of DNA from the retinas of 3-, 10-, 14-, and 20-day-old RCS rats showed only a high molecular weight DNA band. The retinas of rats aged 25, 30, 35, and 40 days showed a ladder pattern of DNA degradation, with bands corresponding to multiples of 180 to 200 base pair subunits (Fig. 1). The ladder pattern was not seen in the retinas of 60-day-old RCS rats or in the normal retinas of Lewis rats.
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FIGURE 1. Agarose gel electrophoresis of retinal DNA. Retinal DNA was isolated as described in Methods and analyzed by electrophoresis through a 1.5% agarose gel. Ten micrograms of retinal DNA was loaded in each lane. Lane 1, molecular weight markers; lane 2, 3-day-old RCS rat; lane 3, 10-day-old RCS rat; lane 4, 14-day-old RCS rat; lane 5, 25-day-old RCS rat; lane 6, 30-day-old RCS rat; lane 7, 35-day-old RCS rat; lane 8, 40-day-old RCS rat; lane 9, 60-day-old RCS rat; lane 10, 50-day-old normal Lewis rat.

In our histopathologic study, 3 days after birth, the retinas of the RCS rats remained immature (Fig. 2A). The thick outer neuroblastic layer had elongated, spindle-shaped nuclei lying perpendicular to the external limiting membrane. Occasional cells in this layer were shrunken with a densified chromatin pattern. Others had relatively pale nuclei with margination of the chromatin material. No outer or inner segments of the photoreceptor cells had yet developed. The TUNEL labeling technique showed few labeled cells, scattered in the outer neuroblastic layer (Fig. 2G). There was no difference in the labeling pattern between the retina at the periphery or the posterior pole.

At 17 days of age (Fig. 2B), the retina had matured. The photoreceptor nuclear layer was approximately 11- to 12-nuclei thick with well-formed inner and outer segments and a developed outer plexiform layer. The TUNEL method failed to show staining of the photoreceptor cells during this period (Fig. 2H). At 20 days, the outer nuclear layer remained of normal thickness, and the outer segments became elongated with an outer zone of irregularly aligned outer segments. Few photoreceptor cells showed labeling with the TUNEL technique. It was not until 25 days of age that the TUNEL technique showed definitive labeling of photoreceptor nuclei (Figs. 2C and 2I).

FIGURE 2. A, B, C, D, E, and F illustrate the pathologic changes of the photoreceptor cells of the RCS rats at 3, 20, 25, 30, 35, and 60 days of age, respectively, in sections stained with hematoxylin and eosin. G, H, I, J, K, and L illustrate the labeling of the photoreceptor nuclei of RCS rats with the TUNEL technique at the corresponding age groups (magnification A and G ×100, other figures ×250). Arrows denote TUNEL-labeled cells.
label was more abundant in the posterior pole than in the peripheral retina. Most of the labeled nuclei were stained with a ring configuration. At 30 days (Figs. 2D and 2J), the outer nuclear layer was reduced to 8 or 9 nuclei in thickness. The outer segments were irregularly aligned with vacuolation in the inner segments. Although most of the photoreceptor nuclei were stained densely with hematoxylin and eosin, a few exhibited shrunken and densified nuclei characteristic of pyknosis. Despite these pathologic changes, few macrophages were noted in the retina. The TUNEL method showed that about one-half of the nuclei were labeled, mostly with a ring configuration around the nuclei. The labeling of photoreceptor cells was more prominent at the posterior pole than at the peripheral retina (Figs. 3A and 3B).

At 35 days of age (Figs. 2E and 2K), the outer nuclear layer at the posterior pole was reduced to 5 to 7 nuclei in thickness. Some of the photoreceptor nuclei cells were densified, whereas others showed margination of the chromatin material. The inner and outer segments were shortened and grossly misaligned. A few degenerated nuclei of the photoreceptor cells migrated into the inner segments of the photoreceptor cells. Few macrophages were seen in the subretinal space. The TUNEL method showed that a great majority of the photoreceptor nuclei were labeled, including those that had migrated to the inner segments. The number of nuclei, labeled homogeneously, had increased.

By 40 to 60 days of age (Figs. 2F and 2L), there was further loss of cells in the outer nuclear layer. A number of macrophages invaded into the outer layers of the retina. They were characterized by large vesicular nuclei distinctly different from those of the photoreceptor nuclei. The outer and inner segment layers had markedly shortened. The TUNEL method showed remarkably few photoreceptor nuclei that were labeled. The labeled cells frequently had homogeneously stained nuclei. The macrophages were not labeled.

To determine quantitatively the extent of programmed cell death in the RCS dystrophic rat retina at different time points, we counted the number of nuclei in the outer nuclear layer of four segments of 0.1 mm length of the sections labeled by the TUNEL technique or stained by hematoxylin and eosin in each eye. Cell counts of two segments were taken on each side of the optic nerve head, one at 2 mm from the ora serrata and one at the posterior pole. The relationship between age and cell counts per 0.1 mm length of sections labeled by TUNEL method or stained by hematoxylin and eosin at the retinal posterior pole was shown in Figures 4A and 4B, respectively. The degenerative process was illustrated by the TUNEL-labeled cells and cell number at each time point. We noted a rapid increase of TUNEL-labeled cells from 25 days and a dramatic loss of photoreceptor cells at the posterior pole between 30 and 60 days of age, when cell number decreased from 90% to 30%.

**DISCUSSION**

In this report, our biochemical study of the RCS rat retina showed double-stranded cleavage of DNA at the linker region between nucleosomes, producing a ladder pattern in agarose gel electrophoresis. In addition, the TUNEL method labeled the nuclear DNA fragmentation in the degenerating photoreceptor cells of the dystrophic retina. Because DNA fragmentation by internucleosomal cleavage is a hallmark of programmed cell death, we concluded that loss of the photoreceptor cells in RCS rats was induced by massive programmed cell death.

The ladder pattern of DNA fragmentation was not seen in the retinas of 3-, 10-, 14-, and 20-day-old rats. This observation corresponded well to the morphologic study, which showed that the photoreceptor cells did not degenerate until the third week of postnatal life. From 25 to 40 days, massive photoreceptor cell loss occurred, and agarose gel electrophoresis of retinal DNA revealed fragmentation of chromosomal DNA, characteristic of programmed cell death. DNA fragmentation disappeared after 60 days, coincident with the extensive disappearance of photoreceptor cells as shown by morphologic study.

The TUNEL method of identifying nuclei with nicked DNA confirmed and extended observations made by electrophoretic analysis of retinal genomic
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DNA. In the immature retina of 3-day-old rats, the presence of scattered labeled cells suggested random and limited programmed cell death during the normal embryonic development of the retina. TUNEL-positive nuclei were not seen in 10-, 14-, and 17-day-old retinas, and few positive nuclei were observed in the 20-day-old retina. Definitive labeling was observed at 25 days. Twenty-nine percent of the photoreceptor cells were labeled in the 30-day-old retina, and 50% of the cells were labeled in the 35-day-old retina at the posterior pole. The TUNEL method allowed us to determine the proportion of cells undergoing programmed cell death at a specific age and also showed the differential programmed cell death between the periphery and posterior pole, with cell death being accelerated at the latter region.

In our morphologic study, a number of histopathologic features characteristically seen in programmed cell death were also noted. We observed that despite the massive loss of cells at 25 to 40 days of age, a minimal macrophage response was seen in the photoreceptor layer. Programmed cell death, an energy-requiring process of self-elimination of cells without production of cellular debris to attract macrophages, provides an explanation for the paucity of macrophages in the early phase of the degenerative process.10 Macrophages, however, were seen clustering around the outer segment debris in the subretinal space at the later stage. Furthermore, one of the characteristics of programmed cell death is the scattered distribution of the targeted cells. In contrast, necrosis inflicted by a noxious stimulus characteristically brings about cell death in cell clusters.10,11 In the early stage of the retinal dystrophy of the RCS rats, the scattered distribution of the TUNEL-labeled photoreceptor cells at 25 days (Fig. 21) was striking.

In disease processes, cell death may occur by necrosis or apoptosis.13 In the RCS rats, we noted a relatively abrupt appearance of TUNEL-labeled photoreceptor cells at 25 to 30 days and a dramatic loss of cells. Gerschenson and Rotello13 postulated that the severity of pathologic stimuli may determine whether cell death occurs by apoptosis or necrosis. The genetic defect in the RPE of RCS dystrophic retina appeared to precipitate programmed cell death in a rapid and massive fashion, and few cells exhibited necrosis characterized by shrunken pyknotic nuclei.

In contrast to the light-induced degeneration of the photoreceptor cells and retinal dystrophy of transgenic mice with a mutant rhodopsin gene in which the retinal degeneration is a primary photoreceptor event, the retinal dystrophy in RCS rats leading to photoreceptor cell death is a secondary phenomenon; the primary defect is a mutant gene in the RPE. The RCS rat provides a model to study various cellular interactions and triggering mechanisms leading to this secondary photoreceptor cell death. The photoreceptor rescue observed in RCS rats in chimeras,3 or brought about by transplantation of RPE from normal rats and by intravitreal injection4 of basic fibroblast growth factors,7 may result from interference of the triggering mechanisms of programmed cell death.

In RCS rats, the programmed cell death exquisitely targets the photoreceptor cells and spares all other neurons of the retina. The external stimuli that activate a gene or set of genes (death genes)13 leading to programmed cell death might vary between the rod and cone populations, between different rod cells in
the outer nuclear layer, and between rod cells in different regions of the eye.

Kerr and colleagues\textsuperscript{8-10} described the light and electron microscopic changes of cells undergoing programmed cell death and termed the process \textit{apoptosis} (from the Greek, "falling off"). They further suggested that perhaps all programmed cell death resulted from apoptosis. Schwartz et al\textsuperscript{15} recently noted that as T lymphocytes and moth intersegmental muscles underwent programmed cell death, they exhibited marked difference in cell surface morphology, nuclear ultrastructure, DNA fragmentation, and polyubiquitin gene expression. The morphologic criteria of apoptosis as defined by Kerr and coworkers\textsuperscript{8-10} did not apply to the intersegmented muscles undergoing programmed cell death. As a result, Schwartz et al\textsuperscript{15} suggested that the terms programmed cell death and apoptosis might not be used interchangeably.

In this article, we prefer the term apoptosis to programmed cell death even though the morphologic features of the degenerating photoreceptor cells did not entirely conform with the criteria of Kerr and coworkers (unpublished observations). However, two well-differentiated cells, such as the photoreceptor and thymocyte, might be expected to have distinct morphologic differences during apoptosis. In addition, our data do not address a condition implicit in the term programmed cell death: that induction of a gene or set of genes such as described for \textit{Caenorhabditis elegans}\textsuperscript{16} is required for the process to occur. Programmed cell death may also imply that cell death occurs by a process independent of the influence of neighboring cells. Photoreceptor cell death in the dystrophic retina of the RCS rat is clearly secondary to a genetic defect in the neighboring retinal pigment epithelium.

In a previous study of light-induced photoreceptor degeneration in rats, we proposed that some of the photoreceptor cells died of apoptosis.\textsuperscript{17} We studied the degenerating photoreceptors with a monoclonal anti-DNA antibody with light and electron microscopy and examined the different stages of nuclear condensation and chromatin margination in the degenerative cells. The chromatin network showed a progressive decrease in the density of DNA labeling. Scattered photoreceptor cells showed cytoplasmic densification. Furthermore, we demonstrated the protective effect of cyclohexamide treatment and suggested apoptosis as one of the mechanisms of cell death in this experimental model. Recently, we reexamined this animal model using gel electrophoresis of DNA and TUNEL technique and found that, under certain experimental conditions, nuclear DNA fragmentation did occur. In addition, we identified TUNEL positive cells in situ in this light-induced photoreceptor degeneration (unpublished data, 1993). Chang et al,\textsuperscript{18} using similar methods, had demonstrated apoptosis in the retinal degeneration (rd) mice, the retinal degeneration slow (rds) mice, and in transgenic mice expressing a mutant rhodopsin gene.\textsuperscript{18} It appeared that apoptosis is not a rare final common pathway of photoreceptor degeneration.

Heintz\textsuperscript{19} postulated that terminally differentiated cells, such as photoreceptor cells, that would not undergo further mitosis may acquire programmed cell death as an alternative effector pathway. He suggested that this pathway may be activated by molecular events that normally lead to transformation of dividing stem cell population and cell growth. We are in the process of examining such molecular events that may precipitate programmed cell death in the photoreceptor cells.

\textbf{Key Words}

RCS rats, retinal dystrophy, programmed cell death, photoreceptor cells, apoptosis

\textbf{Acknowledgments}

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