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An Immunohistochemical Study of an Autosomal Dominant Feline Rod/Cone Dysplasia (Rdy Cats)

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An autosomal dominant, early onset feline model of rod/cone dysplasia has been described. The clinical features, light and electron microscopy, and the electrophysiology were documented. We have now examined in more detail the histopathological and immunohistochemical changes during the early phase of the disease using antibodies against opsin, synaptophysin, glial fibrillary acidic protein (GFAP) and an epithelial marker (MNF118). We have also demonstrated programmed cell death by a modified TUNEL (Terminal deoxynucleotidyl transferase, Uridine triphosphate, Nick End Labelling) technique.

In the Rdy cats, there was significant photoreceptor degeneration between 5 and 17 weeks of age. The TUNEL-labeled cell and pyknotic cell counts in the outer nuclear layer peaked at around 9 weeks of age. Accumulation of opsin in the entire outer nuclear layer of the retina was noted with opsin-immunolabeled rod neurite sprouting. There was a reduction in synaptophysin immunoreactivity in the outer plexiform layer. The Muller cells were activated and expressed GFAP. No significant change of immunolabeling of MNF118 was found. These findings closely parallel those seen in human RP.

Key words: animal model; retinal degeneration; immunohistochemistry; apoptosis.

1. Introduction

Retinitis pigmentosa (RP) is a group of hereditary retinal degeneration characterised by poor night vision and restricted visual fields (Bird, 1995). It has a prevalence of about 1 in 3500 (Berson, 1993) and is one of the leading causes of working age blindness in the developed world (Evans et al., 1996). There is currently no effective treatment.

An autosomal dominant early onset feline model of retinal degeneration has been described previously (Barnett and Curtis, 1985; Curtis, Barnett and Leon, 1987; Leon and Curtis, 1990; Leon, Hussain and Curtis, 1991). Clinically, affected kittens slow dilated pupils and sluggish pupillary light reflexes from 2 weeks of age. Vision is impaired and an intermittent rotatory nystagmus develops between 4 and 6 weeks of age. The first signs of fundal abnormalities are present at 8 to 12 weeks with increased tapetal reflexes. This is accompanied by progressive attenuation of retinal vessels and optic atrophy.

In order to further characterize this model of retinal degeneration, in the current investigation, the immunohistochemical changes in the retina and photoreceptor cell death are studied using markers of apoptosis.

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2. Materials and Methods

Affected kittens ($n = 32$) aged between 10 days to 17·5 weeks were examined with normal littermates ($n = 8$) aged at 10 days to 13·5 weeks for comparison. The animals were bred and studied under the regulation of the United Kingdom Animals (Scientific Procedures) Act 1986 and all animal procedures adhered to the ARVO resolution for the Care and Use of Animals in Vision Research. All animals were killed with an overdose of systemic phenobarbitone, both eyes were enucleated immediately but only one eye per animal was used in this study. After 24–48 h of immersion fixation in 10% formol saline, the inferior-nasal quadrant was embedded in paraffin wax for immunohistochemistry and TUNEL labeling whilst the superio-nasal quadrant was embedded in Araldite resin using standard protocols.

Photoreceptor Cell Counts

Photoreceptor cell counts were performed in 1 μm thick toluidine blue stained Araldite resin sections. A random location near the centre of the section was picked at low magnification. The section was then moved 550 μm from the random spot. An eye-piece graticule (Graticules Ltd, Tonbridge, England) was employed to obtain a retinal strip of 25 μm wide. The number of photoreceptor cells within this strip was counted. The section was then moved 250 μm toward to the centre, and another retinal strip of 25 μm wide was counted. This was repeated twice to obtain a total

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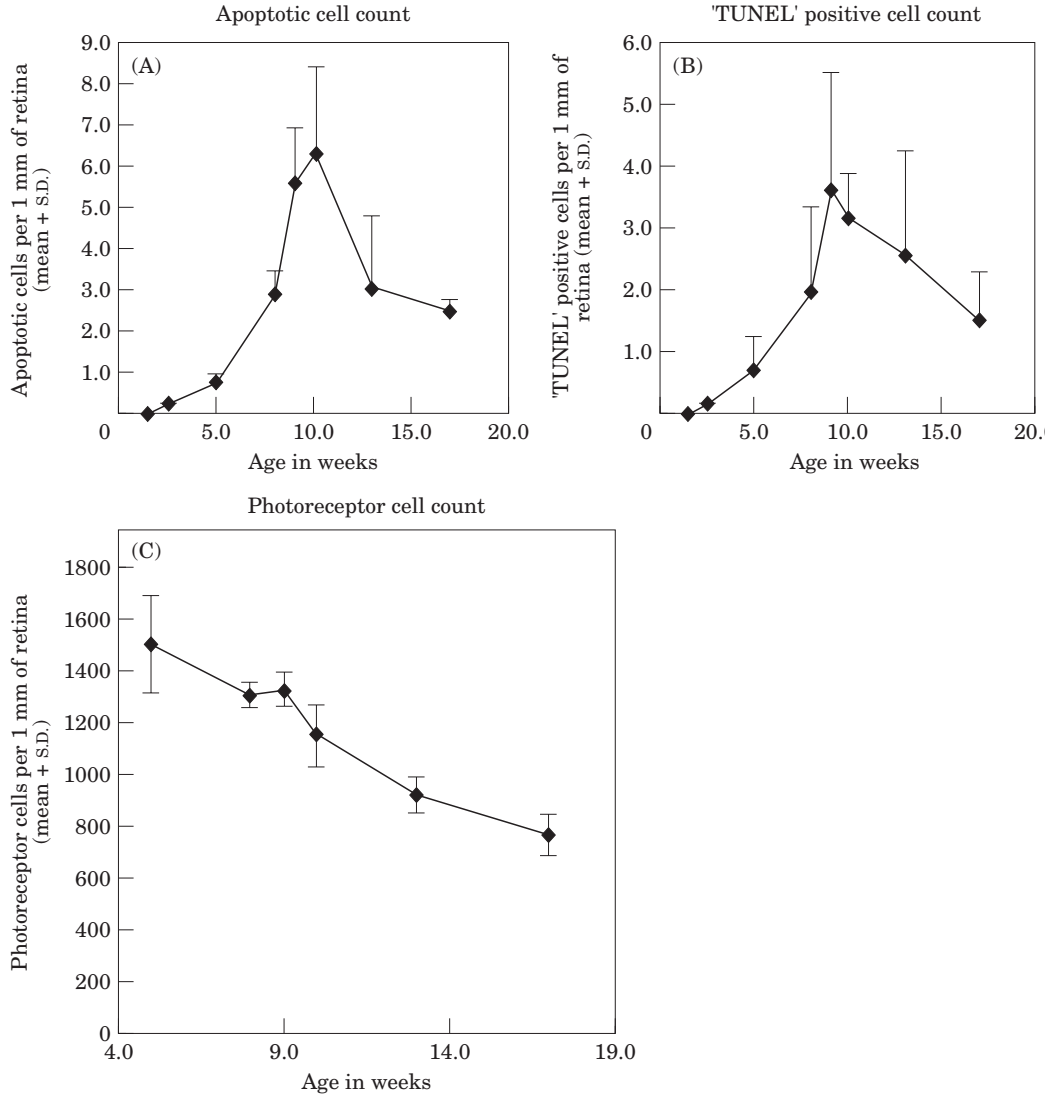

FIG. 1. (A) Pyknotic cell count in the nuclear layer (Mean + S.D.); (B) TUNEL-labeled cell count in the outer nuclear layer (Mean + SD); (C) Photoreceptor cell count (Mean ± S.D.) per 1 mm of retina in the Rdy cats.

of 4 readings. A total of 100 μm of retinal width was counted. The number of photoreceptor cells counted was then multiplied by 10 and expressed as the number of cells per 1 mm of retina.

Demonstration of Programmed Cell Death

The pyknotic appearance and the presence of double strand breaks in DNA suggest programmed cell death (PCD). In this study, we examine these two aspects of PCD in the Rdy cats. A modified TUNEL (Terminal deoxynucleotidyl transferase, Uridine triphosphate, Nick End Labeling) technique was used to detect double strand breaks in DNA. This technique is based on that described by Nakamura and colleagues (Nakamura, Sakai and Hotchi, 1995), but dAdenosine triphosphate replaces dUridine triphosphate. Briefly, the tissue sections were dewaxed with xylene and rehydrated through a graded series of alcohol. Endogenous peroxidase activity was blocked by 0·5% hydrogen peroxide for 25 minutes in the dark at room temperature. Tissue sections were treated with 20 μg ml^{-1} of proteinase K in phosphate buffered saline (PBS) at room temperature for 15 minutes followed by PBS wash. They were buffered with terminal deoxynucleotidyl transferase (TdT) buffer for 10 minutes followed by labeling reaction with 10 mM of dAdenosine triphosphate (dATP), 0·4 mM of biotin 14 dATP and TdT for 60 minutes at 37°C. The positively labeled cells were demonstrated by a standard biotin-streptavidin peroxidase method visualised by diaminobenzidine (DAB). TUNEL-labeled cells were counted in the entire tissue section. The result was expressed as the number of TUNEL-labeled cell per 1 mm of retina.

Pyknotic cells were counted in 1 μm thick toluidine blue stained Araldite resin sections. The result was expressed as the number of pyknotic cells per 1 mm of

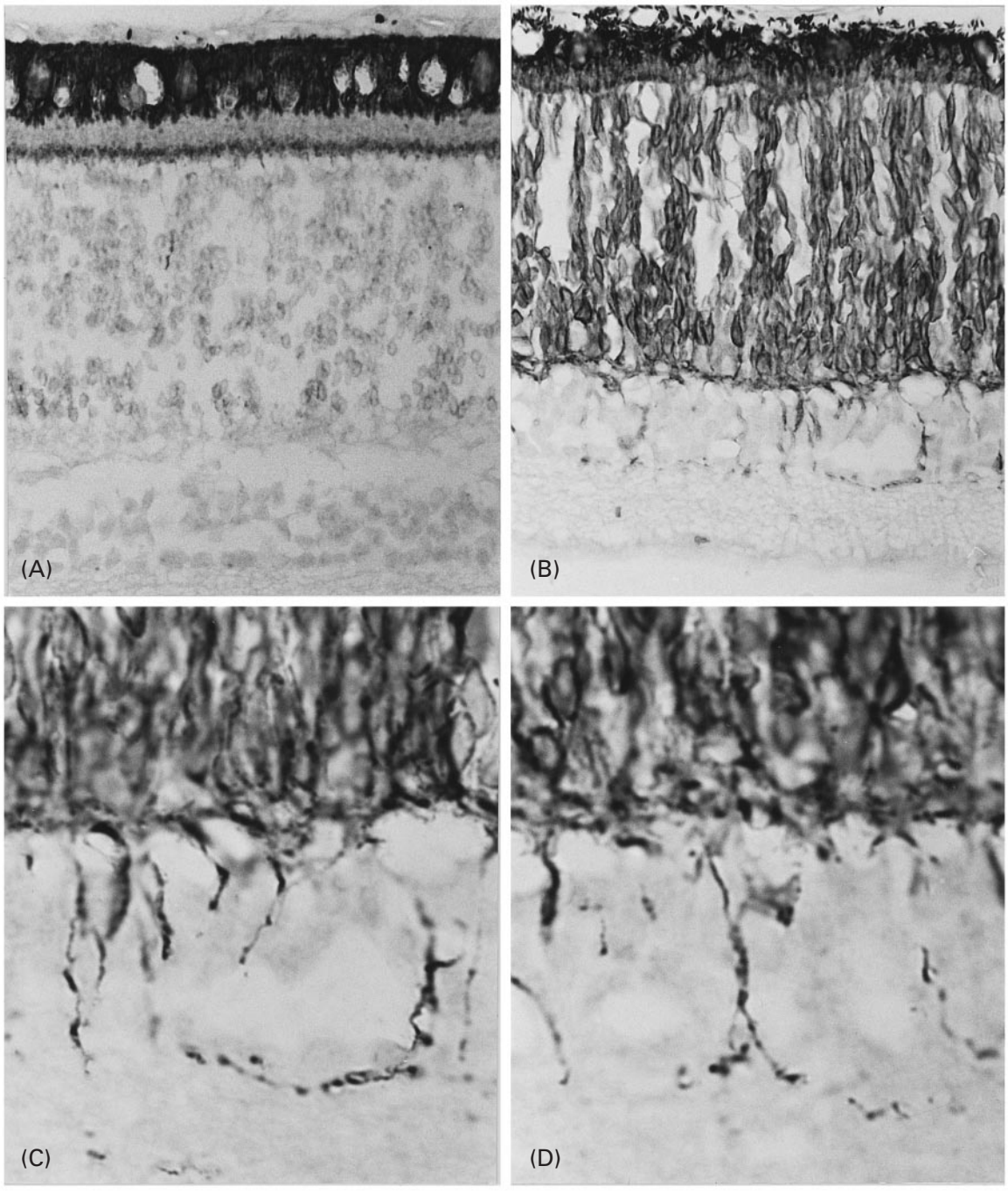

FIG. 2. Opsin immunolabeling. (A) Normal cat at 13·5 weeks × 400; (B) Rdy cat at 13·5 weeks × 400; (C) Rdy cat at 13·5 weeks × 1000; (D) Rdy cat at 13·5 weeks × 1000.

retina. TUNEL-labeled and pyknotic cell were counted by two independent observers and the mean values were calculated.

Immunohistochemistry

The distribution of opsin (antiserum courtesy of Dr D. Bok), synaptophysin, glial fibrillary acidic protein (GFAP) immunoreactivity, and binding of an epithelial marker MNF 118 was investigated using a standard biotin-streptavidin peroxidase method (all antibodies, unless otherwise stated, were obtained from Dako Ltd, England). Antigen retrieval pre-treatment with trypsin was performed prior to all primary antibody incubations (Ordonez, Manning and Brookes, 1988). Appropriate positive and negative controls were used throughout.

3. Results

A small number of TUNEL-labeled cells and pyknotic cells was found in the outer nuclear layer of both normal and affected animals before 5 weeks of age and no significant difference was observed at these time points. In the affected animals, the number of TUNEL-

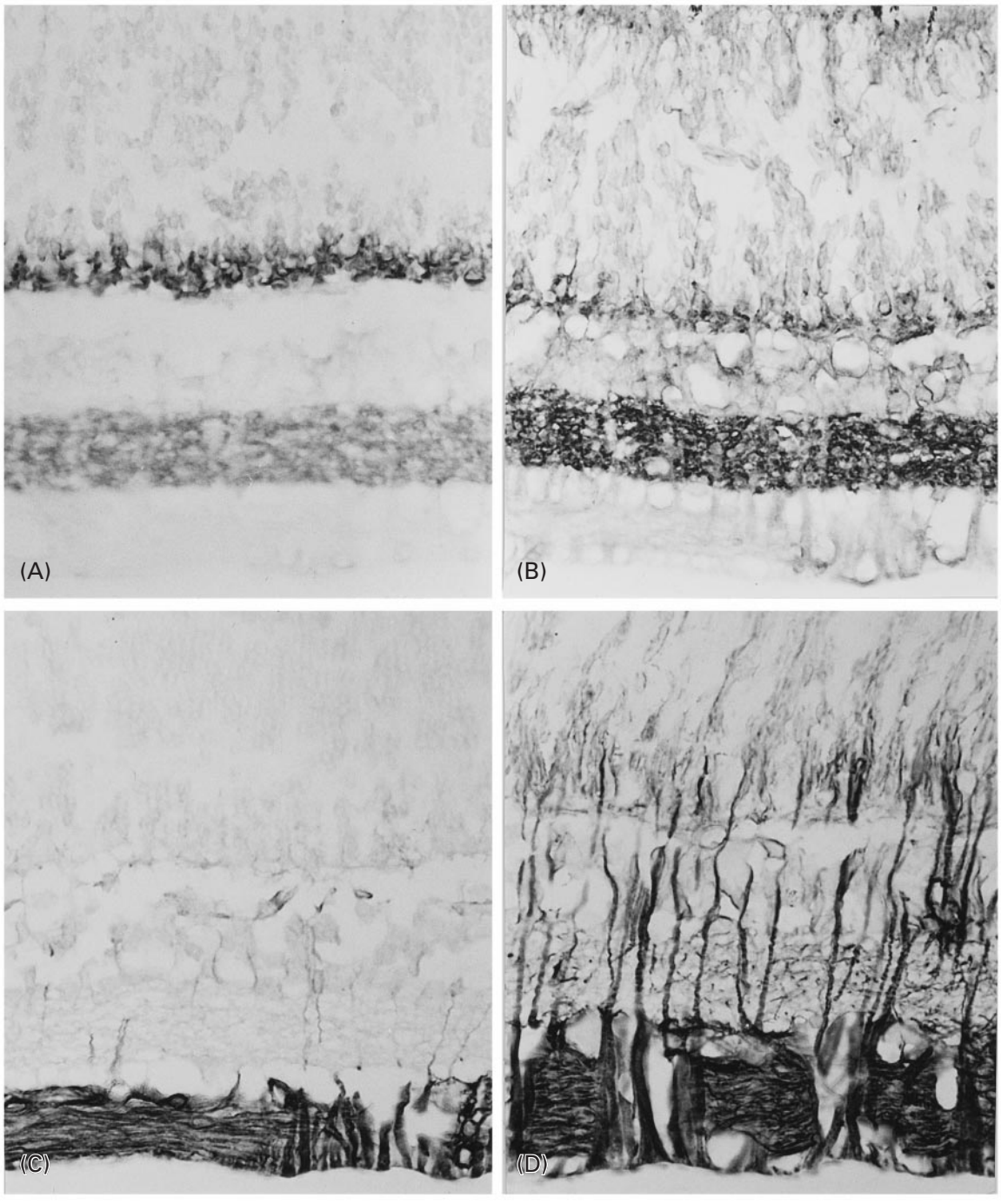

FIG. 3. Synaptophysin and glial fibrillary acidic protein (GFAP) immunolabeling × 400. (A) Normal cat at 13·5 weeks with synaptophysin immunolabeling; (B) Rdy cat at 13·5 weeks with synaptophysin immunolabeling; (C) Normal cat at 13·5 weeks with GFAP immunolabeling; (D) Rdy cat at 13·5 weeks with GFAP immunolabeling.

labeled cells and pyknotic cells increased and peaked around 9–10 weeks of age but none were seen in the controls after 5 weeks. The results of pyknotic cell count and TUNEL positive cell count of the outer nuclear layer (photoreceptors) are summarised in Figs 1(A) and 1(B) respectively.There were no significant differences between the normal and affected animals in the inner nuclear layer and ganglion cell layer of the retina (data not shown). Inter-observer variability was less than 10% for both the pyknotic cell and TUNEL counting. Photoreceptor cell counts showed significant cell loss between 5 and 17 weeks [Fig. 1(C)].

Between 5 and 17 weeks, there was about 700 photoreceptor cells were lost per mm of retina, which was equivalent to an average cell loss of 8·3 cells per day per mm. Over the same period of time, the average TUNEL positive cell count was about 2·5 cells per mm. Hence, the estimated time for the cells to be TUNEL positive was about 8 hr. Using similar calculation, the estimated time for the cells to appear pyknotic morphologically was about 12 hr.

Immunohistochemical staining of opsin was almost entirely restricted to the rod outer segments (ROS) in the normal controls [Fig. 2(A)]. In the Rdy cats, opsin was present in both rod outer and inner segments and there was also accumulation of opsin in the outer nuclear layer and within the presumed photoreceptor cell synaptic terminals in the outer plexiform layer [Fig. 2(B). Some of the rods had sprouted opsin-positive neurites extending towards the ganglion cell layer [Fig. 2(C)] and some had branches [Fig. 2(D)].

Synaptophysin is a synaptic vesicle protein. In the controls, immunolabeling with anti-synaptophysin was largely restricted to the outer and inner plexiform layers [Fig. 3(A)]. In the Rdy cats, the immunolabeling of the outer plexiform layer reduced with age and in parallel with the reduction of photoreceptor cells. No significant change in labeling of the inner plexiform layer with anti-synaptophysin was seen [Fig. 3(B)].

GFAP was expressed predominantly by the astrocytes in the normal controls [Fig. 3(C)]. In the Rdy cats, markedly increased immunostaining of GFAP in Muller cells was observed at all time points [Fig. 3(D)]. Retinal pigment epithelial cell changes were not seen (data not shown).

4. Discussion

PCD is a genetically encoded potential of all cells, and is an essential part of embryonic development, cell turnover and of removal of cells infected by virus or harbouring mutations (Raff, 1992). It is characterised morphologically by disintegration of the nucleolus and generalised condensation of the chromatin, associated with cleavage by an endogenous nuclease of nuclear DNA into short chains of nucleosomes in multiples of 180 base pairs (Gavrieli, Sherman and Ben-Sasson, 1992). Condensed nuclei then fragment, giving rise to apoptotic bodies. In contrast to necrosis, the process affects individual cells within a tissue, neighbouring cells remain healthy, and it takes place in the absence of acute inflammation.

In all models of hereditary retinal dystrophy examined to date, including human RP, cell loss is through the process of PCD (Chang et al., 1993; Portera-Cailliau et al., 1994; Tso et al., 1994). The TUNEL technique has been used to demonstrate PCD in the retinal degeneration (rd), retinal degeneration slow (rds), transgenic Q344ter rhodopsin mutant mice (Chang, Hao and Wong 1993; Portera-Cailliau et al., 1994), RCS rats (Tso et al., 1994) and in human RP (Li and Milam, 1995). It labels the 3′ ends of DNA fragments in nuclei going through the process of PCD in situ (Gavrieli, Sherman and Ben-Sasson, 1992; Nakamura, Sakai and Hotchi, 1995). This technique is more sensitive than the agarose gel electrophoretic analysis of genomic DNA for internucleosomal DNA cleavage (Tso et al., 1994). The nuclei of these cells are, however, TUNEL-labeled for only a short period of time, about 8 h in this model.

It is believed that TUNEL-labeled cells represent an early stage of PCD. This is followed by the condensation of the cytoplasm and nuclear chromatin. In this study, TUNEL-labeled cell counts generally mirrored the pyknotic cell count. It seems, however, that the TUNEL-labeled cell count peaked slightly earlier at 9 weeks as compared to 10 weeks for pyknotic cell count. There were also fewer TUNEL-labeled cells than pyknotic cells.

Although the photoreceptor degeneration starts at about 5 weeks, the maximal photoreceptor loss was between 9 to 13 weeks. PCD was maximal at the onset of maximal cell loss, consistent with our findings. This indicates that PCD is the main mode of cell death in this model of retinal degeneration. As the photoreceptor degeneration presents early, this would be a useful model for the study of PCD manipulation and therapeutic intervention.

Opsin immunolabeling is normally largely restricted to the rod outer segments (ROS). In the Rdy cats, opsin was also present in the rod inner segments and around the cell perikarya. Similar findings have previously been observed in rodent models of retinal degeneration including the rd mice, rds mice, RCS rats and mice with human P23H rhodopsin transgene (Ishiguro, 1987; Nir et al., 1989; Nir and Papermaster, 1989; Roof, Adamian and Hayes, 1994) as well as in human retinitis pigmentosa (Li, Kljavin and Milam, 1995).

In addition to this abnormal perikaryal opsin labeling, the transgenic rhodopsin P23H mutant mouse has accumulation of opsin throughout the outer plexiform layer (Roof, Adamian and Hayes, 1994). This extensive immunoreactivity was not present in the Rdy cats but there were opsin positive rod neurites sprouting towards the ganglion cell layer. This phenomenon has been described in human retinitis pigmentosa (Li, Kljavin and Milam, 1995; Milam, Cideciyan and Jacobson, 1996) but has not been observed in any of the rodent models of retinal degeneration examined to date (Li, Kljavin and Milam, 1995). It has been suggested that the rapid photoreceptor degeneration in the rodents leaves little time for the rod to attempt making new connections, but with slower retinal degeneration in the human, this is a possibility. The precise mechanism of this phenomenon is unknown. It seems likely, however, to reflect changes in the post-synaptic sites on horizontal or bipolar cells. Furthermore, the significance of this phenomenon to the pathogenesis of the disease and its therapeutic implications remain unknown.

The reduction of synaptophysin immunolabeling in the outer plexiform layer of the Rdy cats is also found in other rodent models of retinal degeneration, including the RCS rat (Sheedlo et al., 1993). No significant changes in the inner plexiform layer were expected as there was no significant cell death in the inner nuclear and ganglion cell layers as compared to normal controls, until end-stage disease (Curtis, Barnett and Leon, 1987; Leon and Curtis, 1990). This

finding parallels that observed in moderate human retinitis pigmentosa, where 88% of the inner nuclear layer remained intact (Santos et al., 1997).

GFAP is the major component of glial intermediate filaments found in astrocytes. In the retina, GFAP is present only in astrocytes and Muller cells. However, Muller cells increase GFAP expression in response to focal or generalised retinal injury, such as laser photocoagulation (Humphrey et al., 1993) and light damage (Eisenfeld, Bunt Milam and Sarthy, 1984) as well as in genetically determined retinal degeneration, such as the RCS rats (Eisenfeld, Bunt Milam and Sarthy, 1984), the recessive feline model of retinal degeneration (Ekstrom et al., 1988) and human retinitis pigmentosa (Milam and Jacobson, 1990).

There is evidence to suggest that the increase of GFAP expression is mediated by growth factors. In focal retinal injury, there is a lag period of about 24 hr before a significant increase of expression is seen (Humphrey et al., 1993) suggesting the injury does not directly increase the transcription of the GFAP gene. The delay suggests one or more intermediate steps. Furthermore, the normal area adjacent to the focal injury also has increased GFAP expression. This would be explained by diffusion of one or more growth factors from the site of focal injury (de Raad et al., 1996). This hypothesis is further supported by the increased expression of basic fibroblast growth factor (bFGF) after retinal injury (Wen et al., 1995). Different growth factors can also have different effects on the Muller cells. For instance, intravitreal injection of bFGF induces GFAP immunoreactivity in Muller cells of normal eyes (Lewis et al., 1992) but intravitreal injection of brain derived neurotrophic factor reduces GFAP expression in a feline model of retinal detachment (Lewis and Fisher, 1997).

This glial reaction might reflect attempted photoreceptor rescue during retinal injury. However, these activated glial cells also induce scar formation and might even produce neurotoxins. It would be of great interest to investigate further whether this glial reaction is of benefit to the retina or not.

In comparison to rodent eyes, not only are cat eyes larger in size, the well characterised visual neurophysiology of cats will be of value in the assessment of pathophysiology and efficacy of novel therapeutic agents. The findings in the Rdy cats closely parallel those seen in human RP.

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