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LABORATORY INVESTIGATION

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Apoptosis of photoreceptor cells in ornithine-induced retinopathy

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Abstract ● Background: The intravitreal injection of ornithine produces selective damage to the retinal pigment epithelium (RPE) and results in a loss of RPE, choriocapillaris and photoreceptor cells. To elucidate the mechanism of secondary retinal atrophy, we investigated the presence of apoptotic cells in a rat model of ornithine-induced retinopathy. ● Methods: At 6 and 12 h and 1, 2, 4, 7, 14 and 28 days after an intravitreal injection of L-ornithine hydrochloride in rat eyes, we removed the eyes and subjected them to histopathological examination. We detected apoptotic cells by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate digoxigenin nick end labeling (TUNEL) assay, which stains the 3'-OH ends of fragmented DNA. We used electron microscopy to detect the apoptotic cells morphologically. ● Results: RPE cells were selectively damaged immediately after ornithine administration. TUNEL-positive photoreceptor cells appeared exclusively in the photoreceptor cell layer 12 h after ornithine administration. The number of TUNEL-positive cells increased throughout the 2 days following the injection, then decreased markedly. TUNEL-positive cells remained until 28 days, when the photoreceptor cells had disappeared. The ganglion cell layer, inner nuclear layer and damaged RPE cells were negative for TUNEL staining during all stages. The electron microscopic study also revealed the pyknotic nuclei of apoptotic photoreceptor cells. ● Conclusion: An intravitreal injection of ornithine caused primary damage to the RPE, and subsequently some of the photoreceptor cells revealed apoptosis by TUNEL assay. These findings suggest the dysfunction of the RPE causes photoreceptor cell death according to the intrinsic program of an apoptotic mechanism.

Introduction

The retinal pigment epithelium (RPE) has a major role in maintaining the structure and function of the photoreceptors in the retina. For example, the RPE performs phagocytosis of the outer segment materials and provides substances or enzymes which are necessary for photoreceptor renewal and for the rhodopsin cycle. Its cell membrane has electrical and transport properties that influence the electrical and photochemical activities of the outer segments and that allow the RPE to respond to light. The tight junctions between RPE cells are critical to these electrical and transport properties, and they serve as the "blood-retinal barrier" for the outer surface of the photoreceptors.

Various pharmacological agents are known to affect the RPE cells primarily [10, 17] or to affect the neurosensory retina and damage the RPE secondarily [2, 7, 16]. When the physiological functions of the RPE are altered, the overlying retina is also affected and results in decrease of visual function.

Ornithine is a nonprotein amino acid which plays an important role in the urea cycle. Kuwabara et al. [15]and Ishikawa et al. [12] revealed that an intravitreal injection of ornithine caused severe damage to the RPE and retina. However, these studies could not exclude osmotic effects due to high-dose administration [20], whereas we showed that by intravitreal injection, a small amount of ornithine induced selective damage to RPE without initial effects on photoreceptors [25, 26, 27, 29]. The swelling of cytoplasm and nucleus in RPE cells were the initial changes in the retina, which led to subsequent necrosis and loss of RPE cells, followed by the disappearance of the choriocapillaris and photoreceptors. We speculated that the photoreceptor cell death was caused by apoptosis in ornithine-induced retinopathy.

Materials and methods

Creation of ornithine-induced retinopathy

Eight-week-old Brown-Norway (BN) colored rats were used.

All animals were killed in accordance with the ARVO *Statement for the Use of Animals in Ophthalmic and Vision Research*.

The ornithine solution was prepared by dissolving L-ornithine hydrochloride (Sigma Chemical, St. Louis) in physiologic saline solution and the pH was regulated at 7.2. The concentration of ornithine was 0.25 mol/l (osmotic pressure 550 mosm) and equivalent osmosis of saline solution was used for control study. We injected 0.01 ml of the solution into the vitreous cavity through the equator using a Hamilton microsyringe.

Forty eyes were divided into eight groups of five eyes each (four eyes were injected with ornithine and one was a control) according to the duration after treatment.

The eyes were checked by ophthalmoscopy and fluorescein angiography and were enucleated at 6 and 12 h and 1, 2, 4, 7,14 and 28 days after treatment.

DNA nick end labeling by the TUNEL method

In each of the eight groups, four eyes (three ornithine-injected eyes and a control eye) were used for the TUNEL method [terminal deoxynucleotidyl transferase (TDT) – mediated deoxyuridine triphosphate digoxigenin nick end labeling]. The eyes were fixed overnight in 10% neutral buffered formalin and processed for paraffin embedding.

Sections of 5 μm thickness in the posterior pole of the retina were used for histopathological examination. Formalin-fixed tissues were applied for TUNEL staining using an in situ apoptosis detection kit (Apop-Tag, Oncor, Gaither, Oncor, Gaithersburg, Md.) according to the manufacturer's instructions. Tissue sections were deparaffinized by washing twice in xylene for 10 min and then rehydrated through a graded series of alcohols and phosphate-buffered saline (PBS; 50 mM sodium phosphate, pH 7.4, 200 mM NaCl). Sections were treated with proteinase K (20 μg/ml) for 15 min at room temperature and washed in distilled water. Endogenous peroxidase was inactivated in 3% H_2O_2. Sections were incubated in a moist chamber for 1 h at 37°C with TdT buffer, and for 30 min at 37°C with Stop/Wash buffer. After washing in PBS, the sections were incubated for 30 min at room temperature with anti-digoxigenin peroxidase. The peroxidase label was visualized with 3,3'- diaminobenzidine (Wako Pure Chemicals, Osaka, Japan).

Methyl green was used for the counterstain.

Electron microscopic study

The remaining eight eyes were used for morphologic study. The eyes were processed with 4% glutaraldehyde fixation and 1% OsO_4 post- fixation, and embedded in epoxy resin (Quetol-812: Nissin EM, Tokyo, Japan). Sections of 1 μm thickness in the posterior pole of the retina stained with toluidine blue were used for light microscopic study, and selected areas were used for electron microscopic analysis. Thin sections stained with uranyl acetate and lead citrate were examined under an electron microscope (Hitachi H600, Tokyo, Japan).

Quantitative analysis

Twenty-four rat eyes (three eyes each time at 6 and 12 h and 1, 2, 4, 7, 14 and 28 days after treatment) were subjected to quantitative analysis. The numbers of photoreceptors and TUNEL-positive cells per 0.1 mm length of the section of the outer nuclear layer were counted.

Results

DNA nick end labeling by the TUNEL method

In the control eyes injected by saline solution of equivalent osmosis (550 mosm), microvacuoles were seen in RPE cells and the outer segments slightly disarranged immediately after treatment. No necrotic change occurred in RPE, and microvacuoles disappeared in 2 days. Neural retina appeared normal. No TUNEL-positive cells were seen in the control eyes at any stage (Fig. 1).

Six hours after the ornithine injection, RPE cells were damaged and markedly swollen. No positive cells were detected in the retina by the TUNEL method (Fig. 2). TUNEL-positive cells appeared 12 h after the injection in the outer nuclear layer (Fig. 3). The swollen RPE cells shrank and appeared in irregular shapes at 2 days. The TUNEL-positive photoreceptor cells were most remarkable at 2 days after treatment (Fig. 4). At 7 days, the retinal structure was disorganized. The TUNEL-positive photoreceptor cells had markedly decreased in number. The RPE cells and the outer and inner segments of the photoreceptors had disappeared at 14 days (Fig. 5). TUNEL-positive cells remained until 28 days after the injection, at which time the photoreceptor cells had disappeared (Fig. 6). The ganglion cell layer, inner nuclear layer, and damaged RPE cells were negative by the TUNEL method at all stages.

Fig. 1–6 In situ retinal labeling by the TUNEL method counter stained with methyl green. *Bar* 20 μm ▶

Fig. 1 Twelve hours after the injection of saline solution of equivalent osmosis (550 mOsm) in the rat eye (control). Microvacuoles are seen in RPE cells and the outer segments are slightly disarranged. No TUNEL-positive cells are seen

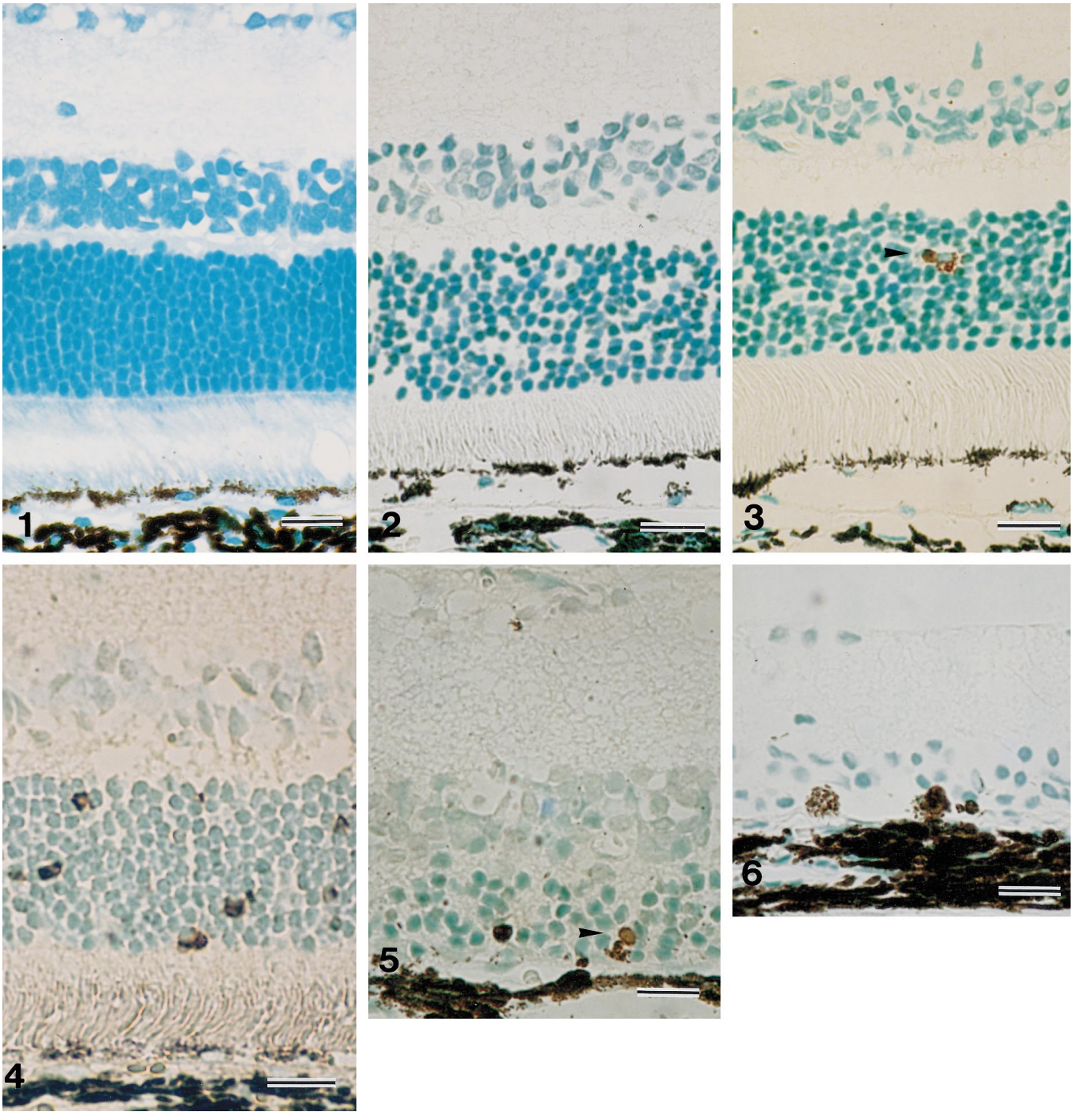

Fig. 2 Six hours after the injection of ornithine solution into the rat eye. Histopathologically, RPE cells are damaged and markedly swollen in the retina. No positive cells are detected by the TUNEL method

Fig. 3 Twelve hours after injection. TUNEL assay reveals a few positive cells (*arrowhead*) in the outer nuclear layer for the first time

Fig. 4 Two days after treatment. Damaged RPE cells have shrunk and the outer segments are disarranged, although the retina has maintained normal structure. A large number of TUNEL-positive cells are seen exclusively in the outer nuclear layer

Fig. 5 Fourteen days after injection. The RPE cells and the outer and inner segments of photoreceptor cells have disappeared. A few TUNEL-positive cells (*arrowhead*) remain in the outer nuclear layer

Fig. 6 Twenty-eight days after ornithine injection. The photoreceptor cells have disappeared, and the inner nuclear layer directly touches on Bruch's membrane. There are some pigment-laden macrophages, but no TUNEL-positive cells are seen in the atrophic retina

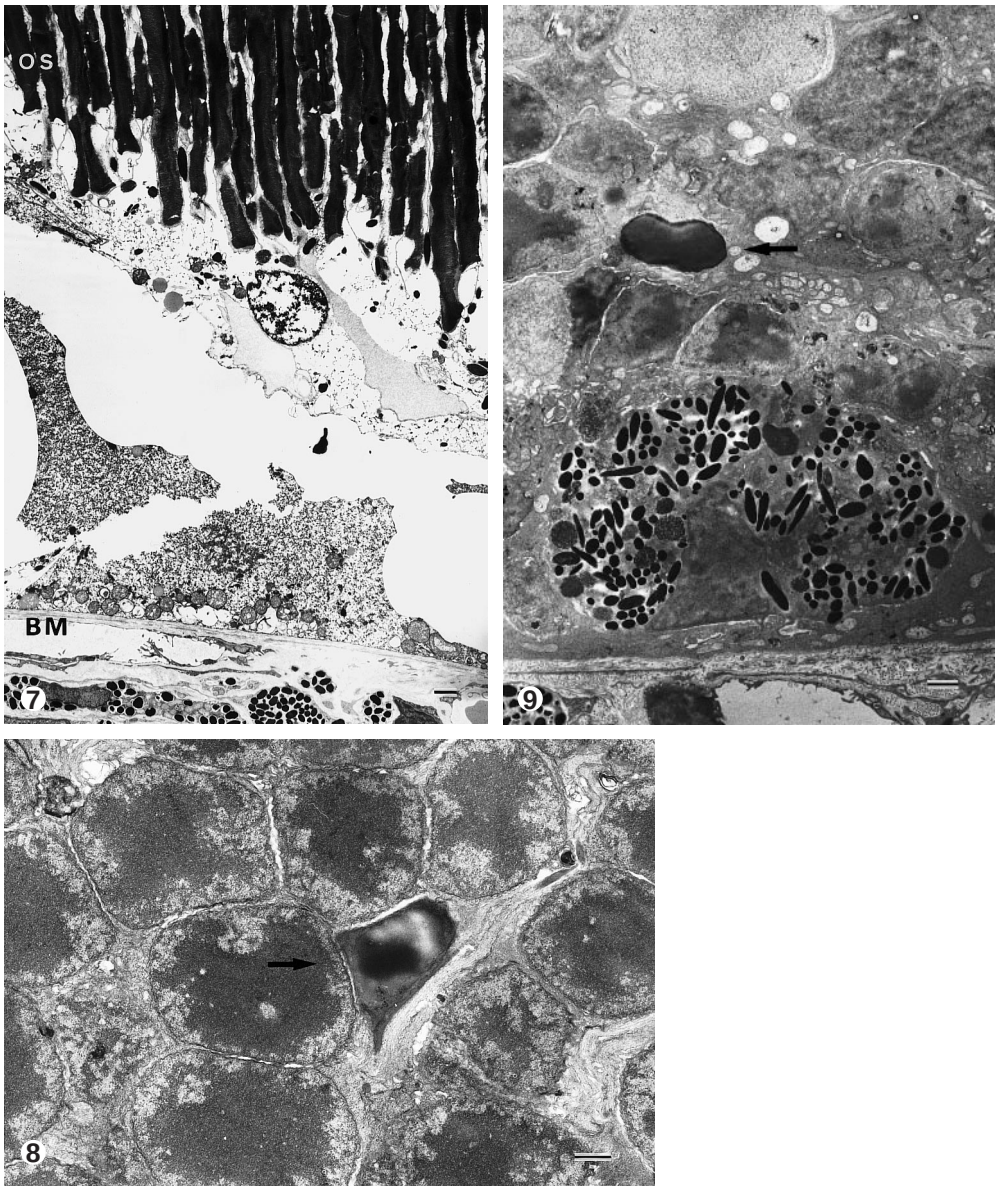

Fig. 7 Electron micrograph of the RPE 6 h after injection of ornithine solution into the rat eye. RPE cells are markedly swollen and cytoplasmic structures have completely collapsed. Melanin granules are irregulary scattered in the cell bodies. *OS* Outer segment, *BM* Bruch's membrane. *Bar* 1 μm

Fig. 8 Electron micrograph of the outer nuclear layer 2 days after injection. There are some shrunken photoreceptor cells (*arrow*) in which pyknotic nuclei are detected. *Bar* 1 μm

Fig. 9 Electron micrograph 14 days after injection. Most of the damaged RPE cells have necrosed and disappaered. There are some macrophages which have phagocytosed many pigment granules. The nuclei of photoreceptors directly touch Bruch's membrane. Pyknotic nuclei are observed (*arrow*). *Bar* 1 μm

Electron microscopic study

The cytoplasmic structures of the swollen RPE cells were completely collapsed 6 h after the injection of ornithine solution into the rat eye. Melanin granules were irregularly scattered in the cell bodies (Fig. 7). In the outer nuclear layer, there were some shrunken photoreceptor cells in which pyknotic nuclei were detected at 2 days after treatment (Fig. 8).

At 14 days, most of the damaged RPE cells had become necrotic and had disappeared. There were some macrophages which phagocytosed many pigment granules. The nuclei of the photoreceptors directly touched Bruch's membrane. Pyknotic nuclei were observed (Fig. 9).

Quantitative analysis

The counts of TUNEL-positive cells were (n=3 in each case); 6 h, 0; 12 h, 1.0; 1 day, 3.7; 2 days, 10.3; 4 days, 6.3; 7 days, 1.3; 14 days, 0.7; 28 days, 0. The rates of TUNEL-positive cells/total photoreceptors were (n=3 in each case): 6 h, 0%; 12 h, 0.4%; 1 day, 1.5%; 2 days, 4.7%; 4 days, 2.1%; 7 days, 1.3%; 14 days, 0.8%; 28 days, 0% (Fig. 10).

Discussion

Apoptosis is a form of programmed cell death defined by characteristic morphological and biochemical changes

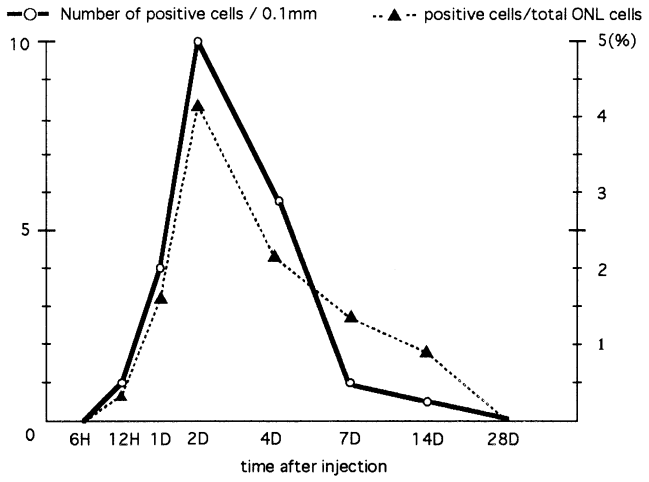

Fig. 10 Quantitative analysis of apoptotic cell death in the outer nuclear layer following ornithine injection. TUNEL-positive photoreceptor cells appeared 12 h after the injection of ornithine solution. The number (average of three eyes, *solid line*) and the rate (TUNEL-positive cells/total photoreceptors; *dotted line*) of TUNEL-positive cells remained at 28 days, at which time the photoreceptor cells had disappeared

[14, 31]. In the retina, apoptosis has been observed in cell differentiation during the normal developmental process. Penfold and Provis [22] described apoptotic cells in various layers of the developing human retina and observed apoptotic bodies within phagosomes of neighboring retinal cells. Additionally, apoptosis has been noted to be a primary mechanism of photoreceptor degeneration in retinal photic injury [1, 18], experimental retinal detachmnt [4] and inherited retinal dystrophy [3, 19, 21, 23, 28] in mice and rats. Studies using murine models of retinal degeneration resembling retinitis pigmentosa showed that initial mechanisms of pathogenesis might be different, but apoptosis was a final common pathway of photoreceptor death in different mutant mice [3].

The TUNEL assay is based on the specific binding of TdT to the 3'-OH ends of fragmented DNA at the single cell level [6], and it stains early-stage apoptosis in systems where chromatin condensation has begun and strand breaks are few, but these precede gross morphological changes.

We have observed a small amount of ornithine-induced necrotic change on retinal pigment epithelium (RPE) selectively in experimental animals [25, 26, 27, 29]. It was of interest that the absence of RPE cells caused loss of the outer and inner segments, as well as the disappearance of the choriocapillaris and photoreceptor cells.

To determine whether photoreceptor cell death in ornithine-induced retinopathy occurred by an apoptotic mechanism, we used the TUNEL method and electron micrroscopic analysis in rat eyes.

In this study, we confirmed that the TUNEL-positive cells were restricted to photoreceptor cells, in which shrinkage and pyknotic nuclei were shown without the involvement of an inflammatory reaction. Electron microscopically, hyperchromatic nuclei were seen, but the morphological features did not entirely fit the criteria for thymocytes laid down by Kerr et al. [14], such as nuclear chromatin condensation at the periphery. A distinct morphological difference may exist between the well-characterized thymocyte apoptosis and photoreceptor cell apoptosis.

The photoreceptor cells showed apoptosis as early as 12 h after the administration of ornithine by the TUNEL method. TUNEL-positive cells were most remarkable at 2 days (up to 5% of photoreceptors) when the morphological changes had not yet been detected in the outer nuclear layer by light microscopy. The number of TUNEL-positive cells increased throughout the 2 days after the injection, then markedly decreased (Fig. 10). TUNEL-positive cells remained when the photoreceptor cells had disappeared. These results are consistent with those of previous studies [1, 4].

It is interesting that the damaged RPE showed no TUNEL-positive staining at any stage after ornithine injection. We speculate that since ornithine causes necrotic change in RPE, the signals for in situ detection of fragmented DNA were negative.

Internucleosomal DNA fragmentation is a hallmark of the apoptotic process [24]. We tried to visualize DNA ladders using an agarose gel electrophoretic analysis of genomic DNA for internucleosomal DNA cleavage visualized by ethidium bromide staining and transillumination with ultraviolet light. Although some low-molecular-weight bands suggesting internucleosomal fragments were observed in ornithine-treated retina, the results were inconclusive, probably because of the small amounts of material available (data not shown). To clarify this point, further studies will be necessary, combined with DNA labeling and Southern transfer to increase sensitivity [30]. Tso et al. [28] reported that TUNEL staining is more sensitive than the agarose gel electrophoresis.

In conclusion, we observed apoptotic photoreceptor cells after an intravitreal injection of ornithine in rat eyes. Ornithine induced primary damage to RPE, and subsequently some of the photoreceptor cells revealed evidence of apoptosis by TUNEL assay. The mechanism responsible for photoreceptor cell death after RPE damage may be as follows: Since RPE cells play an important role in the maintenance of the environment around photoreceptor cells, the dysfunction of RPE causes a disturbance of the photoreceptor metabolism, impaired transport of water and ions, and/or deprivation of trophic factors, such as fibroblast growth factor [8, 11], transforming growth factor [5] and photoreceptor-specific, survival-promoting macromolecular factor, which compose the interphotoreceptor matrix [9].

Apoptosis plays a significant part in some types of atrophy and involution. Kerr et al. [13] reported that atrophy of liver lobes deprived of their portal blood supply was due to massive loss of parenchymal cells through apoptosis.

Our findings suggest that in the process of retinal atrophy induced by ornithine, some of the photoreceptor cells are thinned out according to an intrinsic apoptotic mechanism.

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