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LABORATORY INVESTIGATIONS

Immunohistochemical Study of Apoptosis of Lens Epithelial Cells in Human and Diabetic Rat Cataracts

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Purpose: To evaluate apoptosis of lens epithelial cells by immunohistochemical methods.

Methods: We performed terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assays on capsulotomy specimens (53 patients, 68 eyes) from patients who had undergone cataract surgery, and on the epithelium of diabetic cataracts in rats (72 rats, 144 eyes). An animal model of diabetic cataracts was prepared by injection of streptozotocin in 3-week-old rats. The specimens of rats were also examined using the proliferating cell nuclear antigen (PCNA) immunohistochemical staining method.

Results: Although some TUNEL-positive cells were detected in capsulotomy specimens, we recognized little correlation between their distribution and the morphological classification of the cataracts. In the animal model of diabetic cataracts, TUNEL-positive cells were seen around the regions of accumulated epithelial cells. In the accumulated regions, PCNA-labeled cells undergoing DNA synthesis were also detected.

Conclusions: These results suggest the possibility that apoptosis occurs in human lens epithelial cells. Apoptosis and proliferation of lens epithelial cells may be induced by factors such as hyperglycemia. **Jpn J Ophthalmol 2001;45:559–563**

Key Words: Apoptosis, immunohistochemical staining, lens epithelial cell, TUNEL assay.

Introduction

Recently, apoptosis has been actively discussed in the ophthalmological field. It has been shown that apoptosis occurs in retinal ganglion cells in glaucoma, and in photoreceptor cells in retinitis pigmentosa, and in retinal detachment.[1–3] If apoptosis is involved in intractable diseases for which decisive therapy is lacking, the prevention of apoptosis would inhibit progression or relieve symptoms. Such prevention is a new field, and the study of apoptosis may become of great significance in the ophthalmological field.

This would also apply to the treatment of cataract. Apoptosis during the development/morphogenensis in the crystalline lens has been established, but there are many unclear points and controversies regarding the induction of apoptosis in acquired cataract.[4–12] In this study, we investigated the presence of apoptosis in the lens epithelial cells of humans and in a rat diabetic cataract model using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method.

Materials and Methods

The following two materials were used for apoptosis detection.

Material 1

Human anterior capsules. The study was conducted in accordance with the Declaration of Helsinki, in patients who gave informed consent to this study before surgery (68 eyes in 53 patients). The an-

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terior capsule was incised using a 25-G needle or tweezers during cataract surgery, and immediately fixed in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 2–3 hours. The morphology of the cataract by LOCS III classification and the presence or absence of diabetes were investigated. In LOCS III classification, color and opacity of the nuclei are evaluated in six steps, and cortical and posterior subcapsular opacity are evaluated in five steps by diaphanoscopy, by which cataract progression can be examined in detail.[13,14]

Material 2

Rat diabetes model. All experiments complied with the provisions of the ARVO Resolution on the Use of Animals in Research. Three-week-old male Sprague-Dawley rats (body weight: 50 g) were intraperitoneally injected with 0.1 mg/g body weight of streptozotocin (Sigma, St Louis, MO, USA) to prepare a rat diabetes model. Anterior capsules were excised every other week between 4 and 12 weeks of age. Twelve rats were used each time (60 rats in total). The blood glucose level was measured twice: 1 week after streptozotocin administration and prior to excision of the anterior capsules. Animals with a blood glucose level of 200 mg/dL or lower were excluded. Immediately after the animals were sacrificed by ether anesthesia, the eyeballs were extracted, incised at the posterior lens capsular side, and the anterior capsules with lens epithelial cells were obtained. In the rat diabetes model, immunostaining of proliferating cell nuclear antigen (PCNA), which is an index of nuclear DNA synthesis, was performed. Each sample was fixed in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for the TUNEL method and in methanol for anti-PCNA immunohistochemical analysis.

The anterior capsular specimens obtained were extended on glass slides and treated with 10-fold diluted proteinase K (DAKO, Glostrup, Denmark). ApopDETEK® (DAKO) was used for TdT reaction, and Horseradish Peroxidase-DAB In Situ Detection System® (DAKO) was used for reaction of biotin-labeled dUTP with peroxidase-avidin complex. Immunohistochemical staining of PCNA was performed by the avidin-biotin complex method (ABC method), using Histofine SAB-PO(M) kit® (Nichirei, Tokyo) and anti-PCNA monoclonal antibody (mouse monoclonal antibody NC-012; Novocastra Lab, Newcastle, UK) as the primary antibody. As the negative control in each staining, samples without treatment with TdT and anti-PCNA monoclonal antibody were used, respectively. Fragments were observed under light microscopy via a differential interference prism (Olympus, Tokyo).

Results

Experiment 1

In 59 of 68 eyes, TUNEL-positive cells were observed. Three examples of TUNEL-positive cases are shown below and in Figure 1.

Case A. A 70-year-old man. Diabetes (−), LOCS III: $C_1N_2P_5$
Case B. A 61-year-old woman. Diabetes (−), LOCS III: $C_4N_2P_4$
Case C. A 69-year-old woman. Diabetes (+), LOCS III: $C_3N_4P_2$

The cytoplasm appeared compressed in many TUNEL-positive cells. Significant correlation was not found between the types of cataract and the distribution of TUNEL-positive cells. Although it is not specific to diabetes, the cells linearly disappear in some patients, as seen in case C, and TUNEL-positive cells were observed around the lost cell region in this study. The region of linear cell disappearance covered a width of one to two cells, suggesting that this disappearance was not related to the procedure of using a 25-G needle during surgery.

Experiment 2

In the rat streptozotocin-induced diabetes model, the severity of cataract did not always correlate with the blood glucose level or the period after administration, and it varied greatly among animals. In the transparent lens, TUNEL-positive cells were not observed. In the slightly opaque lens, TUNEL-positive cells were observed as shown in Figure 2A. In the advanced cataract lens, multi-layered epithelium was formed, around which TUNEL-positive cells were observed (Figures 2B, C). In similar regions of multilayered epithelium, abundant PCNA-positive cells were observed (Figure 3). There were no significant changes between TUNEL-positive cells and levels of cataract. However, in rats with advanced cataract, the distribution of TUNEL-positive cells seemed significant around the region of multi-layered epithelium.

Discussion

Based on previous reports, the presence of apoptosis of the crystalline lens cells during development and morphogenesis has been established.[4–6] However, a question arises as to whether apoptosis is in-

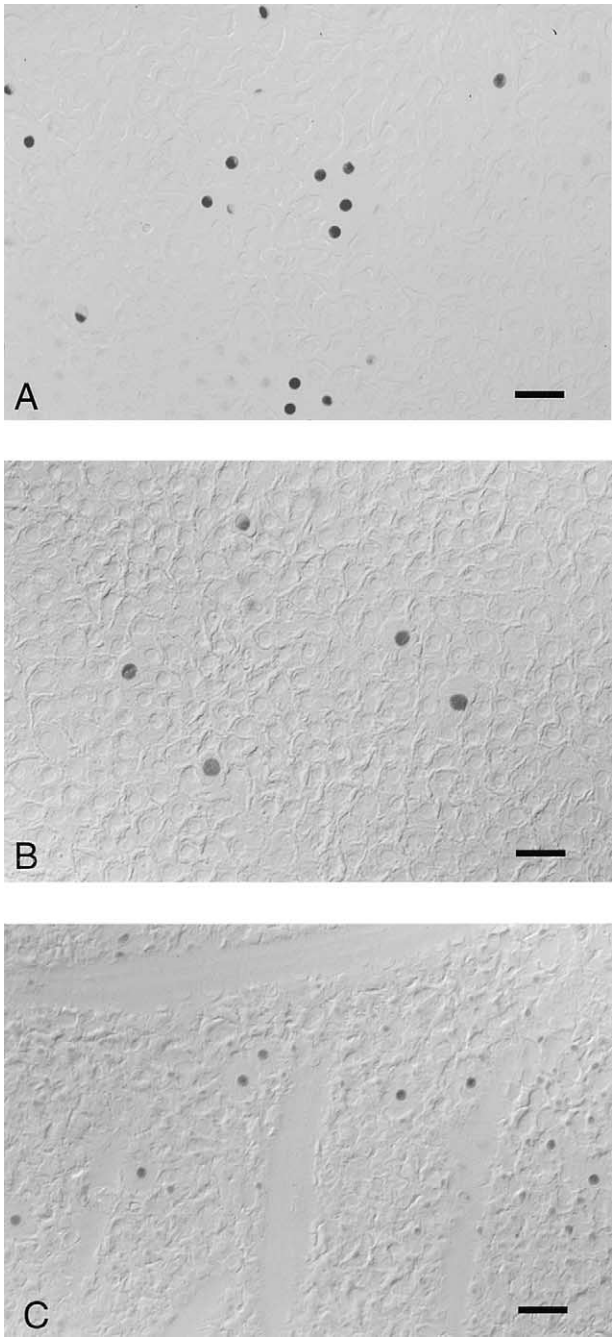

Figure 1. Examples of human anterior lens capsules stained by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling. (**A**) Diabetes (−), LOCS III: $C_1N_2P_5$. (**B**) Diabetes (−), LOCS III: $C_4N_2P_4$. (**C**) Diabetes (+), LOCS III: $C_3N_4P_2$. See text (Results) for details. Bars = 27 μm.

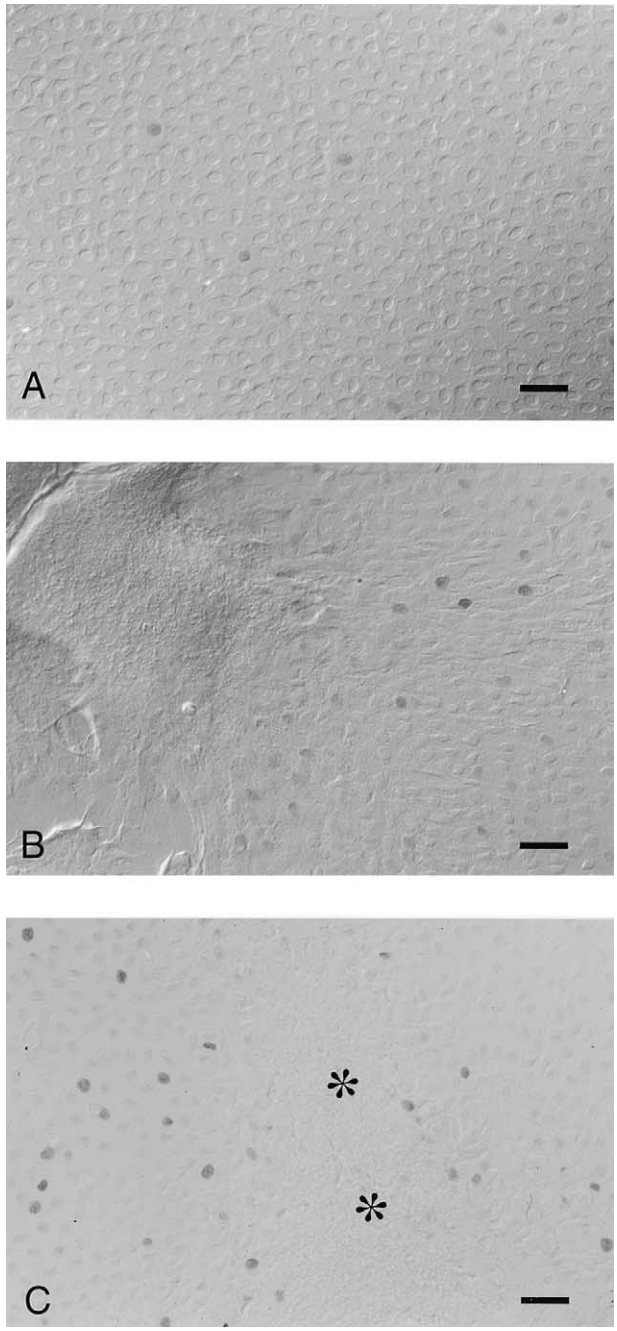

Figure 2. Examples of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining of anterior lens capsules in streptozotocin-induced diabetic rat. (**A)** 6 weeks after administration. Animal showed early cataract. Sparse TUNEL-positive cells were observed. (**B**) 9 weeks after administration. (**C**) 12 weeks after administration. Both (**B**) and (**C**) showed advanced cataract, and TUNEL-positive cells were observed around regions of accumulating epithelial cells (*). Bars = 27 μm.

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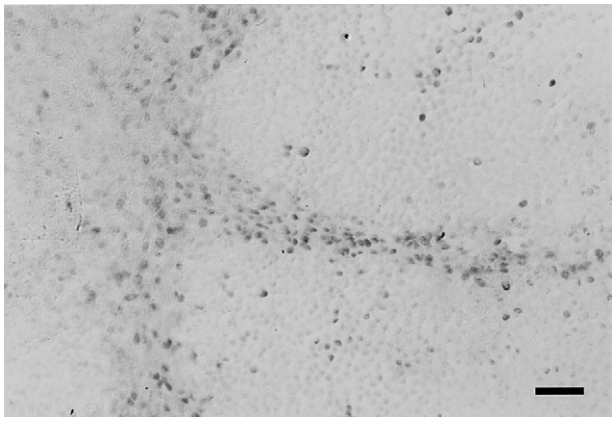

Figure 3. Example of staining of proliferating cell nuclear antigen (PCNA) in anterior lens capsules in streptozotocin-induced diabetic rats. PCNA-positive cells clustered consistently in regions of accumulating epithelial cells. Bar = 55 μm.

duced when cataract occurs by aging or by external stress, such as diabetes, and this issue is still being debated.[8–12] In this study, to obtain information on this issue, lens epithelium was examined by the TUNEL method to investigate the relationship between apoptosis and cataract.

According to the definition of apoptosis, the electron microscopic approach is essential for differentiation, but this method is not appropriate for examination of a wide range of the tissue. Apoptotic bodies can be differentiated from normal nuclei at the light microscopic level, but apoptotic cells in the early stage may be missed. The TUNEL method is very sensitive for detecting the amount of fragmented DNA, and this method can also be applied to fixed preparations. Therefore, this method may be advantageous for investigating the issue described above.[15]

The most important point in discussing the results obtained by this method may be whether positive cells are really apoptotic. It is possible that necrotic cell nuclei is stained.[16–19] Morphologically, both nuclei and cytoplasm are generally swollen in necrosis, while changes in the cytoplasm are considered to be relatively small in apoptosis. However, the cytoplasm appeared compressed in many TUNEL-positive cells in this study. The marginal region of the excised anterior capsular specimens tended to be excessively stained, suggesting that DNA may have been artificially fragmented during the reaction. If only nuclei were stained due to a cause other than apoptosis, these cells may be intermingled in the tissue fragment as false-positive cells. In human anterior capsules, there were no characteristic differences between the presence and absence of diabetes or among the types of cataract. If many false-positive cells were present at the margin of the fragment, it may interfere in the evaluation of the relationship between apoptosis of lens epithelial cells and the degree of cortical opacity. This may have been one reason for missing the relationship between the types of cataract and diabetes. In this study, the number of preparations of diabetic cataract was onl nine, so additional investigation may be necessary to determine the characteristics of TUNEL-positive cell distribution. Although the number of TUNEL-positive cells were not counted, when the duration of fixation was prolonged, especially when the specimens were fixed for a week or longer, the ratio of TUNEL-positive cells to whole fragments appeared to increase slightly. This may be because DNA is fragmented over time in fixed preparations.[20] In this study, the preparations were fixed for 2 hours and stained within 2 days. The findings on observation of the specimens excluding the marginal region were presented. It may be necessary in the evaluation of the TUNEL method to further investigate how differences in conditions such as the duration of sample fixation and concentration of the proteolytic enzyme affect the results.

Assuming that the TUNEL-positive cells observed in this study were apoptotic, such cells would disappear. It is considered that apoptotic cells are generally eliminated rapidly after nuclear aggregation,[21,22] which is the morphological characteristic of cell death. If apoptosis always occurs in lens epithelium, all epithelial cells would disappear in a short time unless the cells are supplemented by new growth. Therefore, some reports question apoptosis of the lens epithelial cells.[9] In the human eye, it is considered that lens epithelial cells proliferate in the germinative zone throughout our lifetime. Cell proliferation in the germinative zone may be sufficient to replace the reduction in the number of cells as the result of apoptosis. Evidence denying apoptosis may not be obtainable because the dynamics of epithelial cells in the germinative zone have not yet been elucidated in detail. Regarding the proliferative capability of lens epithelium, we have investigated mainly in rat sugar cataract by microfluorometry, ^{3}H-thymidine autoradiography, and immunohistochemistry with anti-PCNA antibody, and have confirmed that the degree and distribution of proliferating cells differ by the age of rats and the concentration of galactose in the diet.[22] It is unknown whether apoptosis is first induced and cell proliferation is then induced to supplement the decrease in cells, or inversely, excess proliferation induces apoptosis. Investigations of the proliferative ca-

pability of cells in diabetic rats and apoptosis were independent of each other, and this study was not sufficient to clarify the relationship between proliferative capability and apoptosis. However, the presence of TUNEL-positive cells around the region of multi-layered epithelial cells that are PCNA-positive does suggest a relationship between apoptosis and proliferative ability. By investigating the relationship between apoptosis and proliferation, the mechanism of apoptosis in the lens epithelium may be clarified.

Although causes of cataractogenesis are thought to be varied, it is common for the histological characteristics to be mainly swelling and collapse of lens fibers. Therefore, differences among etiologic causes are unlikely to be reflected in the histological characteristics, which may make the correlation between the distribution of apoptotic cells and disease types of cataract unclear. When exposed to stimulation that causes cataract, apoptosis may be induced in the region that is likely to be affected, resulting in the distribution of TUNEL-positive cells in the region.

It has been shown that in apoptosis, cells signal and control each other's expression.[23] However, the crystalline lens is unique in that it consists of a single cell type that is not affected by blood or lymphatic vessels. Because they can survive independently of signals from other cells, it is considered that lens epithelial cells produce a factor that inhibits cell death.[4,23] If the crystalline lens regulates apoptosis by itself, it is quite possible that changes due to aging and factors such as a high glucose level remove the inhibition and induce apoptosis. Investigation using immunohistochemical methods is useful to clarify the relationship between these etiologic causes of cataract and apoptosis. The study of apoptosis may be important for analyzing the mechanisms of cataracts.

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