



Lens epithelial cell death and reduction of anti-apoptotic protein Bcl-2 in human anterior polar cataracts

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Purpose: In light of the growing body of data implicating apoptosis in cataractogenesis, and in particular, the reported detection of apoptosis in posterior capsular opacification, the purported etiology of which, like that of anterior polar cataracts, is an aberrant transdifferentiation of lens epithelial cells into myofibroblastic cells, we hypothesized that apoptosis could also occur in anterior polar cataracts. Here we sought to examine whether apoptotic cell death occurs in lens epithelial cells from patients with anterior polar cataracts.

Methods: Cell death of lens epithelial cells from anterior polar cataracts, nuclear cataracts, and non-cataractous clear lenses was measured by TUNEL assay and DNA fragmentation assay. The expression of Bcl-2 and Bax was examined using reverse transcription-polymerase chain reaction and Western blot analysis.

Results: Cell death was detected in specimen from patients with anterior polar cataracts by TUNEL assay. DNA fragmentation assay showed the characteristic laddering pattern from the genomic DNA from anterior polar cataracts. The expression of Bcl-2 mRNA and its protein was markedly decreased in lens epithelial cells from patients with anterior polar cataracts.

Conclusions: This study suggests that apoptotic cell death might occur in lens epithelial cells from anterior polar cataracts and decreased expression of Bcl-2 might play a role in the pathologic cellular mechanism of anterior polar cataracts.

The pathologic mechanisms responsible for anterior polar cataracts remain poorly characterized. Aberrant growth and differentiation of lens epithelial cells beneath the anterior capsule of the lens into myofibroblast-like cells has been described and is believed to be responsible for the abnormal accumulation of extracellular matrix molecules [1,2]. The list of such aberrantly accumulated proteins that have been found within the subcapsular plaques includes type I and III collagen, α -smooth muscle actin, and fibronectin [3,4]. More recently, TGF- β -inducible gene h3 (β ig-h3) was shown to be significantly expressed by lens epithelial cells of anterior polar cataract patients [5]. Other studies have also suggested a possible role of TGF- β in cataract formation [4,6,7].

Apoptosis or so called "programmed cell death" plays an important physiologic role in embryonic development, in cellular homeostasis in response to some states of increased cellular proliferation, and in response to various chemical, physical, or biological insults [8]. Although the term apoptosis was originally used to describe a form of cell death distinct from necrosis that may be defined by a set of specific morphologic features including cell shrinkage, condensation of chromatin, and membrane-bound globule formation [8,9], specific bio-

chemical pathways have since been described in great detail. These pathways may be initiated by specific ligand/death receptor, protein-protein interactions, or by other stimuli such as calcium influx, oxidative stress, hypoxia, heat, or ionizing radiation [10]. Moreover, the expression of various apoptotic inhibitors such as Bcl-2 and Bcl-xL and apoptotic promoters such as Bax, Bad, and Bak are important regulators of this process [11].

Apoptosis has been implicated in a host of ocular diseases including glaucoma, retinitis pigmentosa, retinoblastoma, retinal ischemia, diabetic retinopathy, and cataracts [12]. The lens of the vertebrate eye is notable for the presence of only a single layer of epithelial cells at its anterior surface that is believed to be critical in maintaining its transparency [13]. Division of the lens epithelial cells is confined to the lens periphery and as the cells move towards the equator they terminally differentiate into lens fibers. Apoptosis of lens epithelial cells has been noted to occur during this differentiation process. It is also believed that lens epithelial cell death via apoptosis may lead to lens opacification. Previous studies of rat lenses have linked lens epithelial cell apoptosis to oxidative stress [13], UVB irradiation [14], calcium influx [15], and lens extraction (resulting in posterior capsule opacification) [16] and studies of human lens have suggested a role in posterior capsule opacification [17] and in age-related cataracts [13]; although some investigators have challenged the connection between apoptosis and human age-related cataracts [18].

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No systematic studies to date have yet investigated the occurrence of lens epithelial cell apoptosis in human anterior polar cataracts. We first investigated cell death via TUNEL assay and DNA fragmentation assay. The levels of expression of the apoptotic modulator proteins Bcl-2 and Bax were then measured via RT-PCR and western blot analysis.

METHODS

Human specimens: The study was performed according to the tenets of the Declaration of Helsinki, and consents and proper approval was obtained before all experiments.

Lens capsules attached with lens epithelial cells from patients were collected and classified by the same surgeon, according to a modified version of the Lens Opacities Classification Scale (LOCS)-III grading system. Non-cataractous clear lens capsules were obtained during clear lens extraction for the correction of high myopia. The ages of the patients with anterior polar cataract were ranged from 46 to 65 years old and were 50% male. The ages of the patients with nuclear cataract were ranged from 65 to 77 years old and were 40% male. The ages of the control group were ranged from 31 to 45 years old and were 45% male. The anterior capsules were removed and placed immediately into TRIzol reagent (GibcoBRL, Gaithersburg, MD) for RNA preparation, frozen and stored at -70°C for protein extraction, or fixed in neutral-buffered formalin for TUNEL assay. The number of specimen used in each experiment was described in figure legend.

TUNEL assay: Cell death was analyzed using the in-situ cell death detection kit-fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. In short, deparaffinized human lens capsule sections were treated with proteinase K (20 $\mu\text{g}/\text{mL}$, Roche Molecular

Biochemicals, Germany) in 10 mM Tris-HCl (pH 8.0) for 15 min at room temperature. Endogenous peroxidases were inactivated by incubating the sections in 2% H_2O_2 for 5 min at room temperature. Nonspecific antigens were blocked in 2% BSA in PBS for 30 min at room temperature. TUNEL reaction mixture (TdT and fluorescein-labeled nucleotides, 50 μl) was added to each tissue sample and incubated in a humidified chamber for 60 min at 37°C in the dark. A negative control was always included in each batch, and the label solution (without terminal transferase) was applied instead of TUNEL reaction mixture. All sections were counter-stained with 5 $\mu\text{g}/\text{ml}$ propidium iodide (Oncor, Gaithersburg, MD) for 10 min at room temperature. Epifluorescence microscopy (Olympus BX 60F-3, Japan) was used to photograph the propidium iodide staining for both non-apoptotic and apoptotic cells and the fluorescein staining for apoptotic cells.

DNA fragmentation assay: DNA fragmentation analysis was modified from Prigent et al. [19]. Briefly, human lens capsules with attached lens epithelial cells from non-cataractous normal, nuclear cataractous or anterior polar cataractous lenses were homogenized in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and lysed by incubation in the same buffer with 20 $\mu\text{g}/\text{ml}$ RNase A, 75 mM NaCl, 100 $\mu\text{g}/\text{ml}$ proteinase K, and 0.5% Triton X-100. After 3 h incubation at 50°C , the sample was centrifuged for 20 min at room temperature, and the DNA was extracted by phenol purification and ethanol precipitation. Finally, the DNA sample was electrophoresed by 2.0% agarose gel containing ethidium bromide, and photographed under ultraviolet illumination. GeneRuler 100 bp DNA ladder (MBI Fermentas, Hanover, MD) was utilized as DNA size markers.

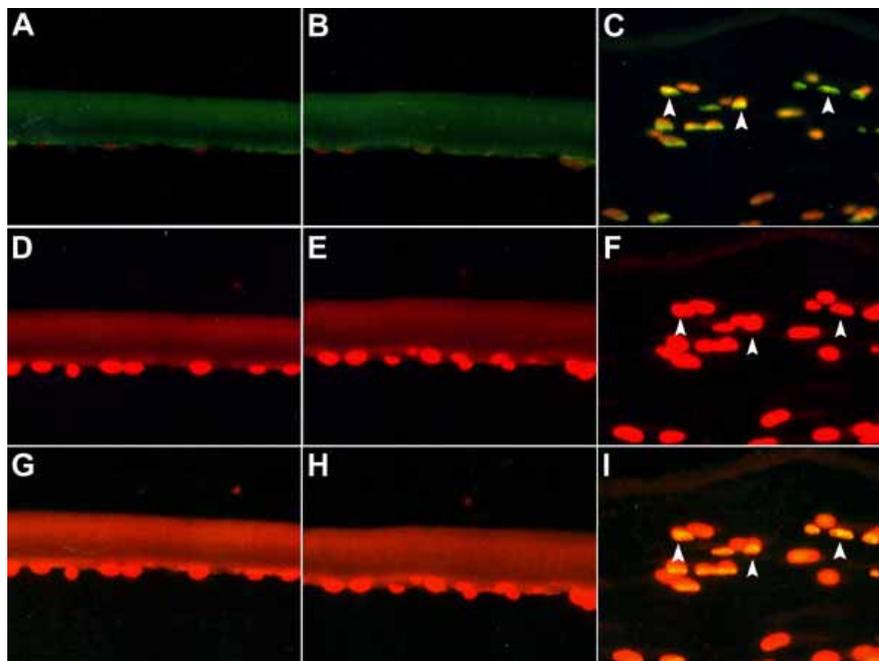


Figure 1. Detection of cell death by TUNEL staining in human anterior polar cataracts. The treated sections were viewed under an epifluorescence microscope. In non-cataractous clear lens, cells were stained with propidium iodide (D) but not with fluorescein (A). There was no co-localization of fluorescein and propidium iodide (G). In nuclear cataracts, cells were stained with propidium iodide (E) but not with fluorescein (B). There was no co-localization of fluorescein and propidium iodide (H). A subpopulation of apoptotic cells, scattered throughout the tissue section from anterior polar cataracts, were intensely stained (bright green to yellow, C) with fluorescein. Cells were counterstained with propidium iodide which stains both apoptotic and nonapoptotic cells (red, F). The co-localization of fluorescein and propidium iodide resulted in a bright yellow mixed color by double filter analysis (I). Arrows point to areas of apoptosis, where fluorescein and propidium iodide were co-localized. The data presented was from one of six independent assays that produced similar results. The original magnification was 200 x.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR): Total cellular RNA was isolated from lens epithelial cells attached to the anterior capsules of human lenses using TRIzol reagent (GibcoBRL) according to manufacturer's protocol. Samples of RNA (1 μ g of total RNA) were reverse transcribed and amplified with gene-specific primers using ThermoScript RT-PCR System kit (GibcoBRL). The primer sequences for the genes and expected product sizes were as follows: 5'-TGC ACC TGA CGC CCT TCA C-3' (sense), 5'-AGA CAG CCA GGA GAA ATC AAA CAG-3' (antisense) for Bcl-2 (293 bp), 5'-ACC AAG AAG CTG AGC GAG TGT C-3' (sense), 5'-ACA AAG ATG GTC ACG GTC TGC C-3' (antisense) for Bax (332 bp), 5'-ATC CCA TCA CCA TCT TCC AG-3' (sense), 5'-CCT GCT TCA CCA CCT TCT TG-3' (antisense) for GAPDH (579 bp). The primers were synthesized by GibcoBRL.

Western blot analysis: Total cell lysates were isolated from lens epithelial cells of human lens capsules by using lysis buffer (25 mM HEPES, pH 7.5, 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.05% Triton X-100, 20 mM β -glycerolphosphate, 1 mM Orthovanadate, 0.5 mM DTT, 0.4 mM PMSF, 2 μ g/ml Leupeptin, and 2 μ g/ml Aprotinin). After centrifugation for 10 min at 12,000 x g, 50 μ g of the total proteins was subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Hybond, Amersham). The membranes were blocked and incubated with mouse anti-Bcl-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-Bax antibody (BD Pharmingen, San Diego, CA), or mouse anti- β -actin antibody (Sigma) at 1:1000 dilution. These blots were then reacted with a horseradish peroxidase-conjugated anti-mouse secondary antibody

(Amersham). A stripping and reprobing method was used between each primary antibody by incubating in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM β -mercaptoethanol for 30 min at 65 °C with agitation. Immunoreactive proteins were visualized by ECL detection reagents (Amersham) on autoradiographic films.

RESULTS

TUNEL assay detects cell death in human anterior polar cataracts: Lens epithelial cell death was investigated via TUNEL staining of anterior polar cataractous, nuclear cataractous, and non-cataractous normal capsule specimens. As shown in a representative experiment (Figure 1), numerous bright green TUNEL positive lens epithelial cells were detected in anterior polar cataract specimens throughout the whole section including cells within the subcapsular plaque. Pale pink spots (Figure 1C) were background labels. These pink spots appeared because of propidium iodide counter staining. The pale pink spots also appeared in control (Figure 1A) and nuclear cataract specimen (Figure 1B). Each of the six anterior polar cataractous specimens was similarly TUNEL positive. By comparison, such TUNEL positive lens epithelial cells were not detectable in all six nuclear cataractous and six non-cataractous clear lens specimens that were assayed.

DNA fragmentation assay confirms cell death in human anterior polar cataracts: To further confirm the occurrence of lens epithelial cell death in human anterior polar cataracts, DNA fragmentation assay was performed using genomic DNA that was isolated and pooled from the portions of the same lens capsule epithelial cells used for TUNEL assay. As shown in Figure 2, DNA isolated from anterior polar cataractous lens epithelial cells, showed the typical laddering fragmentation pattern, usually associated with apoptotic cell death. DNA isolated from nuclear cataractous lens epithelial cells or non-cataractous clear lens epithelial cells showed very little or no DNA fragmentation pattern.

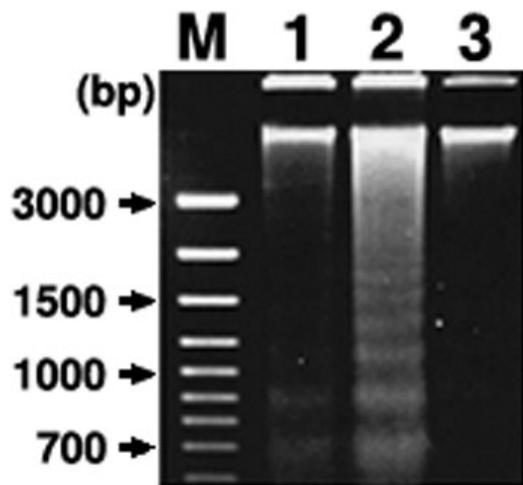


Figure 2. DNA fragmentation assay for human anterior polar cataracts. Total genomic DNA was isolated from lens epithelial cells of patients with nuclear cataracts (lane 1), anterior polar cataracts (lane 2), and non-cataractous clear lenses (lane 3). The three DNA samples were separated in 2.0% agarose gel, stained with ethidium bromide, and photographed under UV illumination. Each genomic DNA was isolated and pooled from the portions of the same lens capsule epithelial cells used for TUNEL assay. Lane M was the 100 bp ladder molecular size marker.

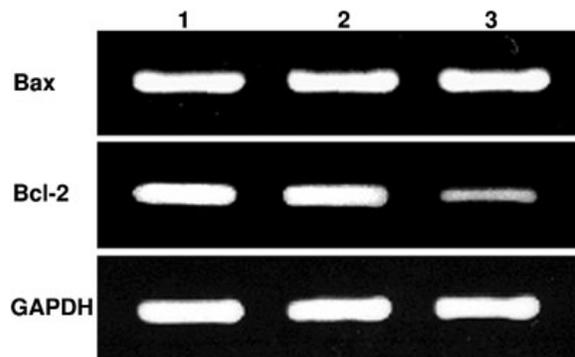


Figure 3. Detection of apoptotic and antiapoptotic markers by RT-PCR. Total cellular RNA was isolated from lens epithelial cells of patients with non-cataractous clear lenses (lane 1), nuclear cataracts (lane 2), and anterior polar cataracts (lane 3). The levels of mRNAs for Bax, Bcl-2, and GAPDH were examined by RT-PCR. The data shown are from one of three independent assays that produced similar results.

Expression of Bcl-2 in patients with anterior polar cataracts: In order to establish a possible altered regulation of apoptotic gene transcription in anterior polar cataracts, RT-PCR was performed on human lens epithelial cell total RNA obtained from patients with anterior polar cataractous, nuclear cataractous, and non-cataractous clear lenses to determine the relative mRNA levels of the anti-apoptotic genes Bcl-2 and pro-apoptotic gene Bax. A complete epithelial specimen was used for each RNA extraction. As shown in Figure 3, the level of Bcl-2 mRNA was decreased in lens epithelial cells obtained from anterior polar cataractous lens capsule compared to both non-cataractous clear lens and nuclear cataractous lens capsule specimens. There was no detectable difference in Bax

mRNA levels between anterior polar cataractous, nuclear cataractous, and non-cataractous clear lens capsule specimens. The amount of GAPDH product, an internal control for PCR amplification, was similar among the samples. The amplified products for Bcl-2 and Bax gene were confirmed by DNA sequence analysis.

To confirm the expression of the corresponding proteins, cell lysates were extracted from the lens epithelial cells attached to the anterior capsules and western blot analysis was performed. A complete epithelial specimen was used for each protein preparation for western blotting. As shown in Figure 4A, the results were in correlation with the RT-PCR data and showed a decreased expression of Bcl-2 protein in lens epithelial cells of anterior polar cataracts compared with nuclear cataracts and non-cataractous controls. Also in correspondence with the RT-PCR data, Bax protein levels were not changed in anterior polar cataract lens epithelial cells compared to both nuclear cataracts and controls. Quantification of each band by densitometric scanning using 6 specimens from each type of patient showed that there was a significantly decreased expression of Bcl-2 in lens epithelial cells from patients with anterior polar cataracts (Figure 4B). There were no detectable differences in Bax protein levels between anterior polar cataract, nuclear cataract, and non-cataractous clear lens specimens.

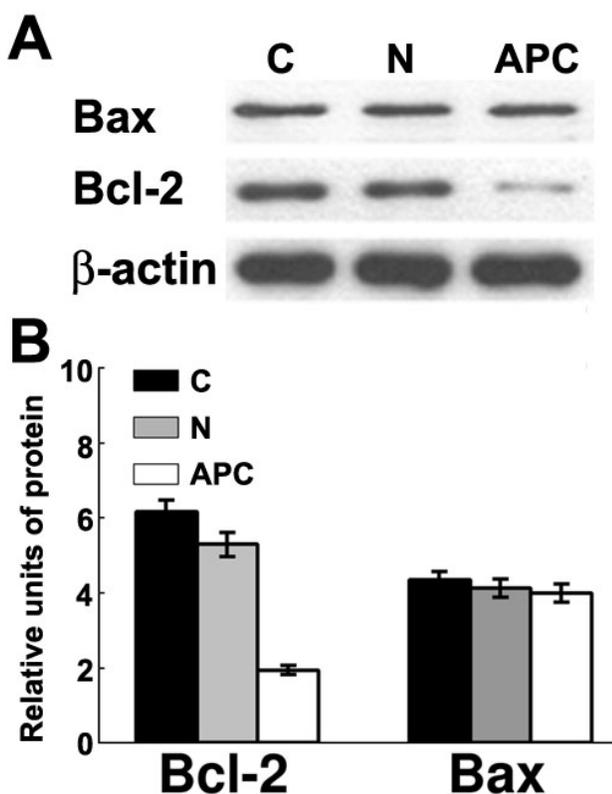


Figure 4. Detection of apoptotic and anti-apoptotic markers by western blot analysis. Cell lysates were prepared from lens epithelial cells attached to the anterior capsules. **A:** The levels of Bax and Bcl-2 proteins were assessed by Western blot analysis. After separating proteins by SDS-PAGE, the whole gel was immunoprobed. β -actin was used as the loading control indicator. Lane C was non-cataractous clear lenses; lane N was nuclear cataracts; lane APC was anterior polar cataracts. **B:** Proteins were quantified using a bioimaging analyzer (Bio-Rad Imaging System, Bio-Rad, Hercules, CA), and expressed in arbitrary units. Proteins in each lane were, control (C), nuclear cataracts (N), and anterior polar cataracts (APC). Each displayed value was normalized against the density of the respective band of β -actin. The bar graph shows the mean value of samples from 6 patients; the error bars indicate the standard deviation. There were statistically significant differences in Bcl-2 levels both between the non-cataractous clear lenses (C) and anterior polar cataracts (APC) and between nuclear cataracts (N) and anterior polar cataracts ($p=0.002$, Mann-Whitney U test).

DISCUSSION

Lens epithelial cells play a vital role in the metabolic homeostasis and maintenance of transparency of the lens [20]. Thus it is thought that damage to lens epithelial cells may contribute to cataractogenesis. Li et al. [13] were the first to possibly demonstrate apoptotic cell death in human cataract specimens (mainly nuclear and cortical). Subsequent studies in animal lens implicated apoptotic cell death as a response to various stressors [13-15] and in secondary cataract formation [16]. Thus an increasingly large body of data is emerging that suggests a role for apoptosis in cataractogenesis.

TUNEL positive staining has been used as an indicator of apoptotic cell death in non-congenital cataract development [13-15]. Harocopoulos et al. [18], however, have suggested that TUNEL positive staining seen in human capsulotomy specimens was a result of necrotic cell death caused by cell damage during surgery. They demonstrated that delay between surgery and fixation might cause necrotic cell death resulting in DNA degradation. In agreement with their observation, we observed little, if any, TUNEL staining in rapidly fixed noncataractous clear lens and nuclear cataractous lens capsule specimens. In comparison, a portion of lens epithelial cells present in anterior polar cataractous lens capsule specimens were still TUNEL positive, possibly implicating apoptotic cell death occurring in lens epithelial cells from anterior polar cataractous lens capsule specimen.

Furthermore, DNA fragmentation assay of genomic DNA obtained from lens epithelial cells acquired from anterior polar cataract specimens revealed the characteristic laddering pattern that has been reported to occur as a result of apoptotic cell death [21]. DNA fragmentation assay of nuclear cataract

samples and non-cataractous clear lens samples showed no evidence of significant DNA degradation via fragmentation assay. The laddering pattern widely reported in the context of apoptosis is believed to be due to internucleosomal Ca^{2+} - Mg^{2+} dependent endonuclease-mediated cleavage. Thus, TUNEL assay and DNA fragmentation assay data demonstrated some modality of cell death occurring in the lens epithelial cells of anterior polar cataracts. However positive TUNEL staining is not necessarily specific for apoptotic cell death and has also been observed with necrosis in some systems [22,23]. Similarly, although initially thought to be specific for apoptotic cell death, the presence of large DNA fragments has also been shown to occur in some forms of necrotic cell death as well [24]. Due to the inherent shortcomings of both of these assays, whether this represents apoptosis or necrosis remains unclear.

Gene expression did, however, appear to be altered in lens epithelial cells of anterior polar cataracts, as demonstrated by decreased level of Bcl-2 in lens epithelial cells of anterior polar cataract compared to nuclear cataract and non-cataractous controls while level of Bax were similar in all three groups. This is the first demonstration of a significant reduction in the level of Bcl-2 in lens epithelial cells of anterior polar cataracts. Bcl-2 is a membrane-bound protein which strongly inhibits apoptosis [25]. Bcl-2 functions as an antiapoptotic protein by forming homo- and heterodimerization with other members of Bcl-2 family of proteins [26]. The ratio of Bcl-2 to Bax is believed to be important in determining cell survival versus cell death [27]. In anterior polar cataractous lens capsules analyzed, this ratio indeed has been altered in favor of apoptosis. Therefore, the reduction in Bcl-2 levels might suggest an "active" means of cell death in lens epithelial cells of anterior polar cataracts.

The significant role of transforming growth factor (TGF)- β in lens pathology has been documented in association with anterior polar cataracts [3,4,28]. It has been previously shown that lens epithelial cells transdifferentiate into myofibroblast-like cells and form fibrotic plaques. In addition to the induction of transdifferentiation, apoptosis of myofibroblast-like cells have been noted. In rat lens epithelial explants, the myofibroblast-like cells induced by TGF- β have been shown to die as the culture progresses in rat lens explant culture system [28,29]. Recent results confirmed apoptotic nuclei in rat lens epithelial explants and whole lenses from rats cultured with TGF- β [30]. Furthermore, in a model of posterior capsular opacification after cataract surgery (after-cataract) that shows similar changes observed in anterior polar cataracts, apoptosis was detected among lens epithelial cells that had undergone myofibroblastic differentiation [16]. The authors suggested that TGF- β in aqueous humor could induce myofibroblastic transdifferentiation and apoptosis in lens epithelial cells after lens extraction. Collectively, these results suggest that TGF- β might be a possible candidate for causing apoptosis in myofibroblast-like cells implicated in the subcapsular plaques. In consistence with these results, our findings in human patients support that cataractogenic properties in anterior polar cataracts might include not only

transdifferentiation of lens epithelial cells but also apoptosis of transformed cells. At present, pathophysiological significance and mechanisms of apoptosis within the fibrotic plaques of anterior polar cataracts are not clear. According to a previous study, a prominent reduction in cellularity occurs during wound healing process [31]. As Maruno et al. [30] pointed out, this reduction in cellularity by apoptosis might happen in the formation of anterior polar cataracts. In respect to Bcl-2 reduction demonstrated in this study, it would be worthwhile to investigate whether TGF- β directly modulates the expression of apoptosis-related genes. An earlier report demonstrated that endogenous survival factors that are secreted by lens epithelial cells held in a tight cell-cell contact influenced the apoptosis in cultured lens epithelial cells [32]. Thus, it could be possible that biochemical changes due to disruption of epithelial nature during transdifferentiation also provide a signal for cell survival and death.

Taken together the data here presented, while not conclusive, are suggestive of the occurrence of apoptotic cell death in lens epithelial cells of anterior polar cataracts. Further studies are needed to elucidate the role of the apoptosis in anterior polar cataract formation and its relevance to TGF- β .

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