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Lens epithelial apoptosis and cell proliferation in human age-related cortical cataract

A. CHARAKIDAS[1], A. KALOGERAKI[2], M. TSILIMBARIS[3], P. KOUKOULOMATIS[1], D. BROUZAS[4], G. DELIDES[2]

[1]Department of Ophthalmology, "Hippocration" General Hospital, Athens
[2]Department of Pathology, University of Crete Medical School
[3]Department of Ophthalmology, University of Crete Medical School, Heraklion
[4]Department of Ophthalmology, University of Athens Medical School, Athens - Greece

PURPOSE. *To probe the presence of apoptosis in the epithelium of human lenses with age-related cortical cataract as well as to assess cell proliferation, a predicted consequence of apoptotic cell death, in this specific cell population.*
METHODS. *DNA fragmentation was assessed using terminal digoxigenin-labeled dUTP nick end labeling (TUNEL) in capsulotomy specimens obtained from patients who underwent either extracapsular cataract extraction for the removal of adult-onset cortical cataract (n=27) or clear lens extraction for the correction of high myopia (n=25). Cell proliferation was assayed in 23 epithelia of cataractous lenses, and 20 epithelia of non-cataractous lenses with the proliferation marker MIB1, a monoclonal antibody against the nuclear antigen Ki-67 that is detected throughout the cell cycle but is absent in the resting (G0) cell.*
RESULTS. *TUNEL staining was observed in 25 (92.6%) specimens of cataractous lenses, whereas cells undergoing apoptosis were identified in 2 (8%) of the epithelia from non-cataractous lenses. Only two MIB1-positive samples were detected, one of which was a capsule obtained during intracapsular cataract extraction.*
CONCLUSIONS. *The epithelium of human lenses with cortical cataract undergoes low rate apoptotic death. This limited epithelial apoptosis is unlikely to result in any significant cell density decrease since epithelial gaps are likely to be replaced by cell proliferation at the germinative zone of the anterior lens capsule. Nevertheless, the accumulation of small-scale epithelial losses during lifetime may induce alterations in lens fiber formation and homeostasis and result in loss of lens transparency. (Eur J Ophthalmol 2005; 15: 212-9)*

KEY WORDS. *Apoptosis, Cataract, Lens, MIB1, TUNEL*

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INTRODUCTION

The anterior surface of the human lens is covered by a single layer of cuboidal epithelium that in the equatorial region of the lens terminally differentiates into the anucleate, elongated, crystallin-rich but organelle-deficient fiber cells that comprise the bulk of the lens mass (1). Unlike the unlimited replicative capacity of the epithelial germinative zone, located in the pre-equatorial region, the cells of the anterior axial part of the lens are considered to be quiescent and not replicative throughout life, bearing a survival mechanism that remains largely unknown. The central lens epithelium is vital for the preservation of lens fiber homeostasis and clarity (2, 3), protecting the underlying fibers

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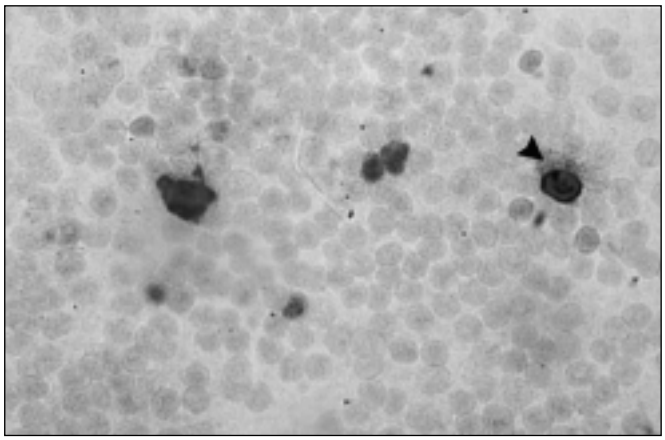

Fig. 1 - *Lens epithelial cells undergoing apoptosis. Apoptotic bodies (arrows). TUNEL x400.*

from injury and oxidative insult (4), transporting fluid and ions to and from the deeper layers of the lens (5, 6) and perhaps providing nutrients to the elongating lens fibers (7). The purported interaction between epithelium and underlying fibers has been well documented (8, 9), although its potential significance in cataract formation has not yet been determined.

Age-related cataract is a multifactorial disease, in which incidence and progress are modified by various factors, including sex, radiation (visible, ultraviolet, and X-ray), oxidation, physical trauma, diet, and medications (10, 11). In view of the important functions attributed to the lens epithelium, epithelial cell death via apoptosis has been implicated in the pathogenesis of both human and experimental animal cataract, with oxidative stress (12), UVB irradiation (13), and calcium influx (14) identified as key promoters of the apoptotic process.

Apoptosis, a form of genetically programmed cell death, is the end result of several biochemical pathways following specific ligand/death receptor and protein-protein interactions, or impact of stimuli such as calcium influx, oxidative stress, hypoxia, heat, and ionizing radiation (15). The cleavage of chromatin into nucleosomal oligomers by increased endogenous endonuclease activity represents the biochemical hallmark of apoptotic death, a process strictly regulated by specific inhibitors, including Bcl-2 and Bcl-xL, and apoptotic promoters such as Bax, Bad, and Bak (16). Morphologically, apoptotic cells feature cell shrinkage, nuclear chromatin condensation, compactness of cytoplasmic organelles, and finally, nuclear and cytoplasmic budding with formation of membrane-bound fragments known as apoptotic bodies (17-20).

Apart from its purported role during development and in maintaining homeostasis in some adult tissues, apoptotic cell death has been implicated, though not conclusively, in several systemic and ocular disease processes, including post-keratectomy haze, corneal lesions, cataract, glaucoma, retinal ischemic retinopathy, and genetic ocular pathologies (21, 22). Although most of these associations, including the detection of apoptosis in the epithelium of human non-congenital cataractous lenses (23, 24), remain controversial or speculative, the detection of apoptosis in the lens epithelium of animals with experimental cortical cataract has been convincingly documented in several studies (13, 23, 25). Based on these data and considering the distinct pathogenesis and biochemical background of human age-related cortical cataract, we thought it important to extend the probe for apoptotic cell death in the epithelium of human cataractous lenses with this specific morphologic type of disease.

In the present study we sought to detect cell kinetics, including both apoptosis and cell proliferation, in lens epithelia collected from patients with age-related cortical cataract, and in a control group of non-cataractous clear lens capsules acquired during lens extraction for the correction of high myopia. We first assayed apoptosis in cataractous and normal lens epithelia by performing *in situ* detection of DNA fragmentation, whereas measuring the incorporation of MIB-1 monoclonal antibody in capsulotomy specimens assessed the proliferative rate.

Experiments were performed in accordance with the Declaration of Helsinki regarding biomedical research involving human subjects. Proper approval was obtained before all experiments.

METHODS

Acquisition of lens epithelia

Capsulotomy specimens were obtained from patients who underwent either clear lens extraction or extracapsular cataract extraction for the removal of adult-onset cortical cataract. In the latter group, the severity of cataract was assessed and classified by the same surgeon, according to the Lens Opacities Classification Scale (LOCS)–III grading system (26).

Following a continuous curvilinear capsulorrhexis, the central 5 to 6 mm of the anterior lens capsule and adherent lens epithelium were withdrawn with forceps. Immediately

upon excision, each specimen was held with fine forceps and washed gently with irrigating solution in order to remove the viscoelastic agent. An initial attempt to fixate the tissue specimens directly in 10% formalin resulted in considerable curling and tearing of the delicate epithelial tags, a technical difficulty also reported in previous studies (27). We opted instead to mount the samples directly on Superfrost glass slides (Menzel-Glaeser, Germany) under the operating microscope and have them "stay afloat" in a drop of balanced salt solution (BSS), ensuring that the tissue specimens were smoothly and evenly spread. Care was taken to place each specimen with its epithelial side up, carefully observing the configuration of the capsulorrhexis and spreading the samples on the glass slide accordingly. After removal of the BSS with a cellulose sponge, the samples were left to air dry for 3 min and then stored in –20 °C, pending further processing. In order to avoid the initiation of epithelial cell necrosis, a key source of false-positive apoptotic staining (28, 29), the time interval between epithelial tag acquisition and storing was reduced to the absolute minimum.

The capsulotomy specimens intended for cell proliferation assay were also collected with the same technique, left to air dry on glass slides, rinsed in acetone for 15 minutes, left to air dry for another 15 minutes, and then stored in –20 °C.

In situ *detection of DNA fragmentation*

The DNA fragmentation was assessed using terminal digoxigenin-labeled dUTP nick end labeling (TUNEL), which is based on the specific binding of TdT to the 3 min -OH ends of DNA, ensuring the synthesis of a polydeoxynucleotide polymer (30). The cryopreserved tissue samples were processed within 48 hours according to the protocol supplied with the *in situ* apoptosis detection Kit-AP (Boehringer Mannheim GmbH, Mannheim, Germany). The air-dried samples were fixed with a freshly prepared paraformaldehyde solution (4% in phosphate buffered saline [PBS], pH 7.4) for 1 hour at room temperature. After rinsing with PBS, the epithelia were incubated in the permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 minutes on ice (4 °C). The slides were rinsed twice with PBS and labeled with the TUNEL reaction mixture (terminal deoxynucleotidyl transferase and fluorescein-labeled nucleotides) for 1 hour at 37 °C in a humidified chamber. The reaction was stopped by rinsing slides three times with PBS, after which the Converter-AP solution was applied on samples. The slides were incubated in a humidified chamber for 30 minutes at 37 °C, rinsed 3 times with PBS, and incubated for 10 minutes at room temperature. Finally, the slides were mounted under glass coverslips (e.g., with PBS/glycerol) and examined under a light microscope (Nikon E400, Japan, 10x40). Very light hematoxylin counterstain was performed before counting. For a negative control, the TdT enzyme was omitted from the labeling reaction in one tissue sample. A lymphoid tissue specimen served as positive control and was processed together with the epithelial tags.

TUNEL-positive cell nuclei were visualized and counted in 40x high-power fields (HPFs) and the number was averaged in five random consecutive fields. Apoptosis was expressed as the number of labeled cells per unit area and as a percentage of the entire cell population.

Cell proliferation assays

Capsulotomy specimens were assayed for ongoing DNA synthesis with the proliferation marker MIB1 (Immunotech, Marseille, France), a monoclonal antibody against the nuclear antigen Ki-67 that is detected throughout the cell cycle (G1, S, G2, M phases) but is absent in the resting (G0) cell (31).

Immunohistochemistry was performed on the air-dried specimens using a dilution of 1:10 of the MIB1 marker with 1-hour incubation period (microwave oven P: 750 W, 3 cycles). A normal lymph node tissue sample and a capsulotomy specimen in which the MIB1 antibody had been omitted from the labeling medium were processed as positive and negative controls, respectively. Following very light hematoxylin counterstain, capsulotomy specimens and controls were examined under a light microscope (Nikon E400, Japan, 10x40). The MIB1 expression was expected to be nuclear and was considered positive regardless of the staining intensity.

Epithelial cell localization

An unexpected finding, observed both in the epithelial tags assayed for apoptosis as well as in those stained for identification of cell proliferation, was the detection of epithelial cells that were detached from the basement membrane and scattered, either single or in clusters, around the borders of the capsulotomy. It was assumed that the epithelial detachment took place during the manipulations for the correct apposition of specimens on the glass slides,

and was not related to the TUNEL or cell proliferation assays. In order to test this hypothesis we sought to identify the position of the epithelial cell population before the initiation of immunochemistry, staining with hematoxylin-eosin a number of epithelial specimens (n = 5) immediately after their mounting on glass slides.

Two epithelial tags were mounted on glass slides without performing any manipulations, regardless of initial orientation and curling, whereas the rest were placed on slides and gently spread and oriented. The air-dried specimens were passed in ethanol 100o for 1 min, rinsed with warm water, and stained with hematoxylin for 1 min (2x). After further rinsing with warm water, eosin staining, and three passages in ethanol, the slides were mounted under glass coverslips (e.g., with PBS/glycerol) and examined under a light microscope (Nikon E400, Japan, 10x40).

RESULTS

In situ *detection of DNA fragmentation*

Twenty-seven capsulotomy specimens of cataractous lenses and 25 epithelia of normal lenses acquired during clear lens extraction for the correction of high myopia were assayed for *in situ* detection of DNA fragmentation (Tab. I). TUNEL staining was observed in 25 (92.6%) specimens of cataractous lenses, which demonstrated an average of 21.7 apoptotic cells per mm^2, or 0.0047% (±0.0027) of the entire cell population (Tab. II). Limited apoptosis (3 apoptotic epithelial cells per mm^2) was detected in 2 (8%) capsulotomy specimens from non-cataractous lenses, a statistically insignificant correlation with the patient group. Although the cataractous lenses demonstrated an expected tendency to progress to morphologically denser forms with increasing age, the apoptotic staining was statistically age- and morphology-independent. Nevertheless, the five higher apoptotic rates were observed in individuals over 70 years of age, with rather morphologically denser cataracts.

TABLE I - PATIENT AND CONTROL GROUP DEMOGRAPHICS

	Patients (cataractous lenses)	Control group (clear lens extraction)
Male/female	22/28	22/23
Mean age, yr (range)	73.4 (50-97)	31.5 (21-47)
Capsulotomy specimens, n	50	45
Apoptosis (TUNEL)	27	25
Cell proliferation (Ki-67/MIB-1)	23	20

The epithelial cells undergoing apoptosis were generally scattered and individual, although a few clusters of apoptotic cells were also detected. TUNEL-positive cells featured cell shrinkage, nuclear — not cytoplasmic — staining, condensed chromatin, lobulated nucleus, with random appearance of various morphologic stages of apoptosis within the cell population (Fig. 1). In a number of both cataractous and clear lenses, weak positive staining was observed around the cut edge of the epithelium or along the border of denuded regions within the epithelial tag, most likely caused by forceps during capsulotomy. This staining was determined to be artifactual, representing DNA fragmentation following physical damage and cell necrosis.

Cell proliferation assay

A total of 43 capsulotomy specimens, including 23 epithelia of cataractous lenses and 20 epithelia of non-cataractous lenses, were processed. Only two MIB1-positive samples (both from cataractous lenses) were detected, one of which was a capsule obtained during intracapsular cataract extraction. This technique, which involves the extraction of the whole lens en bloc, permitted the identification of MIB1-positive cells in the germinative zone of the epithelium, an otherwise normal finding that also served as positive control. Interestingly, the mitotic index in this specimen was determined to be 0.007%, which is within the range of the proliferation rate (0.005% [±0.002]) expected in the germinative epithelium of normal lenses (32). A faint staining observed in several specimens was considered to be background, since it was also present in specimens in which the MIB1 antibody had been omitted from the labeling medium.

Epithelial cell localization

Epithelial detachment was not observed in tissue specimens that were mounted on glass slides without further manipulations. These epithelial tags, however, were found wrinkled or incorrectly orientated, and were determined unsuitable for further processing. On the contrary, those mounted on glass slides and carefully spread and oriented were found to be surrounded by epithelial cells, either single or in small clusters, that represented roughly 0.05–0.1% of the entire cell population and presumably resulted from the subtle rubbing of the tag against the glass slide. Since

the tissue specimens were immediately air-dried and stored in –20 °C, the short time interval between the mechanical stress and the deep freezing of the epithelial tags was considered unlikely to result in the initiation of the apoptotic process, an assumption further substantiated by the absence of TUNEL-positive cell nuclei among detached cells in capsulotomy specimens from non-cataractous lenses. These specimens were therefore determined suitable for processing and were included in the study.

DISCUSSION

Cell death via apoptosis in lens epithelial tags

The detection of apoptosis in the epithelium of human non-congenital cataractous lenses has been controversial. Li et al were the first to support a potential link between lens epithelial cell apoptosis and cataract formation in humans and animals, investigating the effect of oxidative stress on lens epithelial integrity and demonstrating the apoptotic death of epithelial cells with subsequent lens opacification (23). These conclusions, however, were discredited by Harocopos et al, who suggested that the TUNEL staining observed in capsulotomy specimens from cataractous lenses is rather false-positive and presumably results from epithelial cell necrosis induced, in part, by cell damage during surgery (24). They also hypothesized that the detected DNA fragmentation could also be secondary to necrotic cell death associated with the delay between surgery and fixation of tissue specimens. Subsequent studies, nevertheless, have reported the detection of lens epithelial cell apoptosis in human anterior polar (33), atopic (34), posterior subcapsular (35), and hyperglycemia-induced cataract (36) as well as in experimental animal models of cataract induced by selenite (37), staurosporine (38), hyperglycemia (39), interferon-gamma (40), and UV irradiation (41). This growing body of literature implicates a poten-

TABLE II - DETECTION OF APOPTOSIS (TUNEL assay) IN THE PATIENT GROUP

Patient	Age, yr	Cataract morphologic classification	Cells/mm^2	Apoptosis, %
1	75	C4	35	0.008
2	97	C4	14	0.003
3	69	C3	19	0.004
4	71	C4	51	0.011
5	69	C5	33	0.007
6	75	C4	22	0.005
7	88	C5	9	0.002
8	79	C3	23	0.005
9	76	C3	47	0.010
10	60	C4	21	0.005
11	70	C3	0	0
12	50	C3	5	0.001
13	64	C4	25	0.005
14	77	C4	28	0.006
15	83	C3	14	0.003
16	80	C4	9	0.002
17	76	C5	19	0.004
18	71	C1	24	0.005
19	75	C3	23	0.005
20	74	C3	0	0
21	73	C5	17	0.004
22	59	C3	19	0.004
23	61	C4	23	0.005
24	85	C3	14	0.003
25	78	C4	42	0.009
26	77	C4	31	0.007
27	69	C3	19	0.004

tial link between apoptosis of lens epithelial cells and loss of homeostasis in the layer of lens fibers with subsequent lens opacification.

Our findings indicate that small-scale loss of epithelial cells via the apoptotic pathway is present in human lenses with cortical cataract, expanding the growing body of relevant literature (33-36).

The identification and differentiation of apoptosis from necrotic cell death was based on the detection of both the immunohistochemical as well as the morphologic hallmarks of apoptotic death: nuclear TUNEL staining, condensed chromatin, lobulated nucleus, and asynchronous appearance of different stages of apoptosis in the population of stained cells. It is intriguing that these findings correlate well with observational data from the pre-apoptotic era, which referred to the presence of dysfunctional cells in the epithelium of cataractous lenses, bearing morphologic features similar to cells that we would nowadays describe as apoptotic.

Karim et al observed vacuoles, pattern irregularities, and clefts in the epithelium of lenses containing subcapsular and cortical opacities (32), whereas Straatsma et al noticed various epithelial cell abnormalities, including crenated nuclear profile, cytoplasm engorged with multiple abnormal-appearing Golgi figures, lysosomal bodies intruding into lucent intracellular cisternae, and extensive extracellular lacunae (42).

Accurate mounting on glass slides and rapid fixation of the tissue specimens was critical for the correct interpretation of our findings. Smooth spreading and orientation of the capsulotomy specimens on the glass slides enabled the reliable staining of the entire cell population, though at the cost of limited epithelial detachment. In addition to the impact of the mechanical strain, early loss of interdigitations between lens epithelial cells undergoing apoptosis, an observation recently confirmed by electron microscopy (13), may also account for the observed fragility of some distinct cells.

Since DNA degradation by released endonucleases in cells undergoing necrosis has been implicated as a potential cause of false-positive TUNEL staining (28, 29), rapid fixation of our specimens was essential in order to avoid the initiation of the necrotic process. Noteworthily, immunohistochemical detection of necrosis does not exclude the presence of apoptosis, since in improperly fixated tissue specimens apoptotic bodies may undergo secondary necrosis in vitro and, thus, be a source of false-positive necrotic staining.

Lens epithelial cell proliferation and impact on cell density

The density of the lens epithelial cell population, a parameter that obviously depends on the equilibrium between epithelial cell loss and respective cell proliferation, has been estimated to be stable in human cataracts of various forms and degrees of severity (23, 24), although this finding has not been unequivocal (27). Unlike the high rates of apoptosis detected in tumors, the apoptotic index of the lens epithelium, a normal – not cancerous – tissue, would be expected to be low. In our study, lens epithelial cells were estimated to undergo apoptosis at a rate of 0.0047%, considerably less than the 4 to 40% rate reported by Li et al (23) and even smaller than the postulated mitotic index of the germinative zone of the lens epithelium (0.005% [±0.002]) (32). The latter finding implies that, apart of other pathophysiologic consequences, cataract-related apoptosis of the lens epithelium is unlikely to result in lens epithelial depletion. The exact recovery mechanism of the central lens epithelium following cell damage remains uncertain, with either cell movement from the germinative zone or proliferation of adjacent epithelial cells (43) being the likely alternatives. Our observation that the central zone of the anterior lens capsule displays no detectable mitotic activity may suggest that the average of 21.7 cells/mm^2 lost by apoptosis in this region are likely to be replaced by slow migration of cells originating in the proliferative pre-equatorial zone of the epithelium. Further substantiating this hypothesis, the transcription factor activator protein 1 (AP-1), an essential part of the replicative activation mechanism of the epithelial cells during lens wound repair, has been found to be fully functional only within lens cells of the equatorial zone (44). In addition, studies on the effect of oxidative stress on lens epithelial cells showed that the stress and proliferation-related protein proliferating cell nuclear antigen (PCNA) remains constant following epithelial cell death, indicating no detectable change in proliferation (45).

Although the replacement of central epithelial defects is most likely accomplished by dividing cells from the germinative zone, the redirection of pre-equatorial epithelium towards the central anterior lens surface has been found to induce changes in the bow of differentiating equatorial cells (13). Noteworthily, Karim et al observed an abnormal pattern of cell density in the epithelia from lenses with cortical cataract, in that cell density decreased rather than increased from the center to the pre-equatorial zone (32). These observations indicate that apoptosis, either of the

central or the peripheral anterior epithelium, directly affects cell population kinetics and disturbs lens fiber formation.

Lens epithelial cell apoptosis and cataract formation

The connection of lens epithelial damage with the compromise of the underlying lens fibers' survival and clarity has been established in several studies (13, 23, 25) that attribute cataract formation to lens epithelial cellular or biochemical alterations. UVR threshold exposure of the rat lens epithelium, for example, has been shown to induce subepithelial opacities that belatedly follow lens epithelial cell apoptosis and are restricted to the fibers differentiating at the moment of exposure (13). Although the detection of the exact mechanism of cortical cataract formation following epithelial apoptosis was beyond the scope of this study, we assume that several alternative or complementary mechanisms may be involved. Critical alterations in the structure of differentiating lens fibers, either due to redirection of dividing pre-equatorial cells towards the central anterior lens surface or initiation of the apoptotic process in the germinative and equatorial zones of the lens by the same stimuli that induce apoptosis in the central lens epithelium, may accumulate during lifetime, even at a very slow rate, and eventually contribute to the formation of cortical cataract. Moreover, the apoptotic death of distinct lens epithelial cells may induce the compromise of the permeability barrier function of the anterior lens capsule with end result the loss of homeostasis of the underlying lens fibers. This pathophysiologic mechanism was first identified in the intestinal epithelial monolayer where both spontaneous and induced apoptosis of isolated epithelial cells was found to hollow out the intestinal barrier and facilitate the loss of solutes and the uptake of noxious agents, even though the entire cell population remained stable (46).

CONCLUSIONS

Low rate apoptosis was detected in the epithelium of human lenses with cortical cataract. The observed apoptosis is unlikely to result in any significant epithelial cell density decrease since lost cells are continuously replaced by cell proliferation at the germinative zone of the epithelium. Nevertheless, the accumulation of epithelial losses during lifetime may induce alterations in lens fiber formation and homeostasis by several potential mechanisms: loss of the epithelial permeability barrier and direct insult of subepithelial fibers, apoptotic loss of germinative and differentiating equatorial epithelial cells by the same factors that induce apoptosis in the central region, and derangement of lens fiber formation due to the redirection of germinative cells towards the central region for the replacement of epithelial cells lost by the apoptotic pathway.

Reprint requests to:
Antonios Charakidas, MD
16b Hierou Lochou
Byron-Athens 162 33, Greece
acharakidas@yahoo.com

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