Apoptotic Cell Death in Rabbit Lens After Lens Extraction

Katsuhiko Kato,* Daijiro Kurosaka,* and Toshiyuki Nagamoto†

**Purpose.** To determine whether apoptosis or necrosis of lens epithelial cells occurs after lens extraction.

**Methods.** Lens extraction was performed on 24 rabbit eyes. The authors then performed terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and transmission electron microscopy on the eyes 1 to 10 days after surgery.

**Results.** The postoperative lens was demarcated into three regions by the adhesion between the incised edge of the anterior capsule and the posterior capsule: the adhesive region, the intracapsular region, and the central portion of the posterior capsule. By day 2, TUNEL-positive cells with morphologic characteristics of necrosis were detected in the intracapsular region. Beginning on day 5, TUNEL-positive cells with morphologic characteristics of apoptosis and necrosis were detected in the adhesive region. Apoptotic cell death in this region was detected among the myofibroblast-like cells. Lens epithelial cells that had extended onto the central portion of the posterior capsule began to diminish on day 8 or 9, some of them morphologically demonstrating necrotic changes.

**Conclusions.** Apoptosis of lens epithelial cells occurs in the process of wound healing and reepithelialization after lens extraction, leading to secondary cataract. If the induction of apoptosis is better understood, protocols might be developed that could prevent reepithelialization through apoptosis, thus delaying or preventing secondary cataracts. Invest Ophthalmol Vis Sci. 1997; 38:2322-2330.
correlates with contractility of LECs within the gels. Additionally, TGFβ induces myofibroblastic transformation and apoptosis in cultured rat LECs.20

As outlined above, wound healing after lens extraction occurs around the site where the incised edge of the AC adheres to the PC. The number of myofibroblast-like LECs in this region decreases gradually after initial proliferation.10 We recently reported that in aphakic rabbits, α-SMA-positive cells that had migrated onto the central area of the PC abruptly disappeared by postoperative day 10.24 From these results we hypothesized that cell death, especially apoptosis, brings about cell reduction in the adhesive region and on the PC. Because myofibroblast-like LECs appeared from 5 days after surgery and disappeared on the central PC by day 10,24 we studied cell death in rabbit lens 1 to 10 days after surgery using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL)27 and transmission electron microscopy.

METHODS

Surgical Procedure

Twenty-four young female Japanese albino rabbits, weighing approximately 2.5 kg, were anesthetized by intravenous injection of sodium pentobarbital (25 mg/kg; Abbott Laboratories, North Chicago, IL). Eye drops of 0.3% norfloxacin (Banyu, Tokyo, Japan), 0.1% sodium diclofenac (Wakamoto, Tokyo, Japan), and 0.5% tropicamide containing 5% phenylephrine hydrochloride (Santen, Osaka, Japan) were instilled into the eye before surgery. A 3.2-mm superior limbal incision was followed by a continuous curvilinear capsulorhexis on the center of the AC, and the lens material was removed with a phacoemulsifier (Cavitron 9001, Alcon Surgical, Fort Worth, TX). The limbal incision was closed with a shoelace 9-0 nylon suture. At the end of the surgery, 12 mg of tobramycin (Shionogi, Osaka, Japan) was injected subconjunctivally, and oxytetracycline-polymixin B ointment (Pfizer, New York, NY) was applied topically. All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Tissue Preparation

On postoperative days 1, 2, 3, 5, 7, 8, 9, and 10, the eyes were examined with slit lamp microscopy. At each time point, three rabbits were killed by an intravenous overdose of sodium pentobarbital. The eye subjected to surgery was enucleated and fixed immediately in 10% neutral buffered formalin. The eyeball was cut in half approximately 3 mm posterior to the limbus, the anterior half was embedded in paraffin in the usual manner, and 2-μm tissue sections were prepared for the TUNEL assay.

TUNEL Assay

The TUNEL assay was carried out as described by Gavrieli et al27 with some modifications. In brief, deparaffinized tissue sections were treated with proteinase K (20 μg/ml, Dako, Carpinteria, CA) in 10 mM Tris-HCl (pH 8.0) for 8 minutes at room temperature (RT). Endogenous peroxidases were blocked by incubating the sections in 0.3% H2O2 in methanol for 20 minutes at RT. The sections were preincubated in TdT buffer (30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) for 10 minutes at RT and then incubated with 0.5 unit/ml of TdT and 40 μM biotin conjugated 11-dUTP in TdT buffer for 60 minutes at 37°C in a humid chamber. The reaction was terminated by immersing the sections in SSC buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 minutes at RT. After washing with phosphate-buffered saline (PBS), background staining was blocked with nonimmune goat serum for 10 minutes at RT. The sections then were incubated with streptavidin–peroxidase (Zymed, South San Francisco, CA) for 10 minutes at RT, rinsed three times in PBS, and stained for 6 minutes at RT using 3, 3′-diaminobenzidine, tetrahydrochloride (Wako, Osaka, Japan). After stopping the reaction with water, counterstaining was performed with methyl green (Chroma, Kongen, Germany) or hematoxylin (Zymed). Rabbit small intestine was used as a positive tissue control. In addition, one section of each sample was incubated with 1 μg/ml of DNase I in TdT buffer for 10 minutes at RT before incubation with TdT and biotinylated dUTP as a positive reaction control. TdT, biotin 11-dUTP, and DNase I were purchased from Enzo Diagnostics (Farmington, NY) and stored at −20°C until use.

Transmission Electron Microscopy

The entire postoperative lens was taken from eyes on days 9 and 10 and cut in half in PBS. One half was prepared for the TUNEL assay as described above. The other half was prepared for transmission electron microscopy. The tissue was immediately cut into small pieces, fixed in 2% glutaraldehyde (Nacalai tesque, Kyoto, Japan), postfixed in 1% osmium tetroxide (Taab, Berkshire, United Kingdom), and embedded in Epon (Oken, Tokyo, Japan) by the usual procedure. After observation of semithin sections stained with toluidine blue, ultrathin sections were prepared and stained with uranyl acetate and lead citrate and observed through a transmission electron microscope (HU-12AS, Hitachi, Japan).

RESULTS

TUNEL Assay

Findings for the eyes are described for the adhesive region, the intracapsular region, and the central por-

Adhesive Region. On postoperative day 1, LECs had migrated from the AC onto the PC through the adherent zone of both capsules. On day 2, LECs were accumulating around this adherent zone, but no TUNEL-positive cells were detected until day 3. LECs in this region were transformed into spindle-shaped cells, with scattered TUNEL-positive cells being detected among them on and after day 5 (Fig. 3). On light microscopy these cells exhibited characteristic morphologic features of apoptosis, such as a translucent halo around a condensed nucleus.

Intracapsular Region. As shown in Figure 4, some of the LEC underlying the AC were TUNEL positive on postoperative day 1. Stained cells were in contiguous groups, and morphologically they demonstrated swelling of the nucleus and cytoplasm, characteristic of necrosis. Such TUNEL-positive cells, consistent with necrotic death, were detected only until day 2. On day 3, LECs on the peripheral portion of PC began to elongate and transform into lens fiber cells. Elongation continued throughout the experimental period, especially near the equatorial region, and the ring of Soemmerring had formed in some eyes by day 10. However, no TUNEL-positive cells were detected.

Central Portion of the PC. No cellular components were observed in this region until postoperative day 3. On day 5, flattened cells began to extend onto the central portion of the PC from the capsular adhesive region (Fig. 5A). Cell migration continued; the migrating cells were recognized as a faint opacity of the PC through a slit lamp microscope (Fig. 5B). On day 8 or 9, the opacity abruptly regressed, suggesting an abrupt decrease in cells (Fig. 5C). No TUNEL-positive cells were detected throughout the experimental period (Figs. 5A, 5D).

Transmission Electron Microscopy

Because the characteristic structure of apoptosis was detected in the adhesive region only after day 5 under light microscopic level, we performed electron microscopic study on the lens on days 9 and 10. Characteris-
tics of apoptosis were demonstrated ultrastructurally among the LECs in the adhesive region on day 9 (Fig. 6). Necrotic and mitotic changes also were observed in the same region (Fig. 7). Other viable cells in this region showed morphologic features consistent with myofibroblastic transformation (see Figs. 6 and 7). LEC on the central portion of the PC showed necrotic changes (Fig. 7C).

**DISCUSSION**

In this study we demonstrated that apoptosis occurs in the wound healing process after lens extraction, leading to secondary cataract. Apoptosis was detected only in the adhesive region where the incised edge of the AC adhered to the PC.

To determine the manner of cell death, morphologic examination by the TUNEL assay and electron microscopy was used. TUNEL, widely used to detect apoptotic cells, does not always discriminate between apoptotic and necrotic death. In that respect, corroborating examinations, such as morphologic study by light and electron microscopy, are helpful.

The earliest death of LECs in the intracapsular region was observed on day 1 and exhibited characteristic features of necrosis. However, apoptotic and necrotic cells were detected in the adhesive region from days 5 to 10. Ishibashi et al. have shown that degeneration of LEC occurred in the same lesion but late after surgery. These authors depicted disruption of the plasma membrane, translucent cytoplasm, and cellular debris, all of which typify necrotic death. However, apoptosis takes only hours to occur, and its detection...
FIGURE 6. Transmission electron microscopic findings on day 9. (A) An apoptotic cell (arrow) and an apoptotic body (arrowhead) among the lens epithelial cells near the wrinkled posterior capsule. Other viable cells exhibited elongated cell bodies, convoluted nuclei, and numerous protrusions on the cell surface, all of which are consistent with myofibroblastic features. (A, upper inset) A slit lamp photomicrograph taken soon before sacrifice showed fibrous opacification around the incision of the anterior capsule (arrowheads). (A, lower inset) A light photomicrograph with toluidine blue staining showed accumulation of lens epithelial cells around the edge of the anterior capsule. The posterior capsule demonstrated obvious wrinkles. (B) Higher magnification of the apoptotic cell in A. Typical chromatin...
FIGURE 6. (Continued) condensation (arrows) was observed within a separated or lobulated nucleus. Condensation of the cytoplasm was also observed. Note the subplasmalemmal microfilaments with dense bodies characteristic of myofibroblasts (asterisks) within adjacent viable cells, and extracellular tonofilibrils (tf). (C) Higher magnification of the apoptotic body in A. A lysosome within a lens epithelial cell contained nuclear chromatin (nc). The nucleus of this cell (N) seemed to have a normal appearance. Abundant cytoplasmic microfilaments with dense bodies (asterisks) and secretion of tonofilibrils (arrows) were also observed. (D) Another apoptotic cell presenting massive condensation of the nuclear chromatin (nc) within the whole nucleus. This cell also contained subplasmalemmal microfilaments.
FIGURE 6. (Continued) (arrowheads). A desmosome-like structure (arrow) was observed between this apoptotic cell and an adjacent cell. ac, anterior capsule; pc, posterior capsule. Bar = 1 mm (A, upper inset), 100 μm (A, lower inset), 10 μm (A), 1 μm (B, C, D).

in tissue sections is difficult when a small number of cells is involved.28,33,34 In addition, the final result of apoptosis is the formation of small apoptotic bodies that in vivo are rapidly phagocytosed by neighboring cells or by macrophages. Apoptotic bodies that are not ingested eventually lyse in a process similar to necrosis. Therefore, the cell degeneration observed by Ishibashi et al could have included some obscured element of apoptosis.

FIGURE 7. Transmission electron microscopic findings of the same eye as in Figure 6. (A) A necrotic cell in the adhesive region exhibiting swelling of the nucleus (N) and disruption of the plasma membrane (arrow). (B) A mitotic figure with a centriole (arrow). Note the extracellular fibrils (asterisk). (B, inset) Higher magnification of extracellular fibrils demonstrated bands with a periodicity of approximately 50 nm, indicating collagen fibrils. (C) A necrotic cell on the central portion of the posterior capsule showing swelling of the nucleus (N), disruption of the plasma membrane (arrow), and translucent cytoplasm. pc, posterior capsule. Bar = 10 μm (A), 1 μm (B, including inset, and C).

The elongation of LECs and the formation of the ring of Soemmerring in the intracapsular region represent differentiation of LECs into lens fibers.56 Although apoptosis is involved in the terminal differentiation of lens fiber cells,58 no apoptotic cells could be detected in the intracapsular region. It is possible that apoptosis had not yet occurred early in the postoperative period being investigated. Because no TUNEL-positive cells were detected in this region on and after day 3, we did not perform electron microscopic observation in this area.

In the central portion of the PC, we expected that cell disappearance was caused by apoptosis because it occurred abruptly without remarkable inflammatory signs; apoptosis was not detected. However, this interpretation deserves further investigation, because apoptotic cells lose their ability to maintain cell-to-cell or cell-to-matrix contact early in the cell death process.28 Accordingly, apoptotic cells would be likely to detach from the central portion of the PC, leaving no evidence of apoptosis.

LEC's in the adhesive region, which present mesenchymal morphologic features of myofibroblasts, exert contractile force on the lens capsule, and produce extracellular matrix.11,12,24 underwent apoptotic death. Because these cellular activities resemble those of granulation tissue myofibroblasts in the skin,14,16,18,19 we believe that their roles in intraocular wound healing resemble those of granulation tissue myofibroblasts. A difference also was evident between wound healing of the skin and that of the lens. Cell reduction in lens wound healing occurred by both apoptosis and necrosis, but reduction of granulation tissue myofibroblasts was caused by apoptosis, not necrosis.16,18 This difference presumably results from anatomic and environmental differences. The lens is a unique tissue that lacks vascularity, and its wound healing does not involve angiogenesis. In addition, LECs are nourished through the aqueous humor, which has an osmolarity lower than that of serum or intracellular fluid.86 Therefore, LECs directly exposed to the aqueous humor exist in an environment unfavorable to survival and become sensitive to cytotoxic stimuli. Although further investigations are required, we speculate that the necrosis observed in this study results from such conditions.

Recently, as the apoptotic process has been investigated, its relations with the pathogenesis of various diseases have been elucidated.37-39 This enabled trials to be conducted of treatments designed to alter the apoptotic process in diseases such as cancer, leukemia, and acquired immunodeficiency syndrome.90,91 In the lens, if the induction of apoptosis of LECs is better understood, then proto-
Apoptosis After Lens Extraction 2329
cols might be developed to prevent reepithelialization through this process, at the apoptotic step, and secondary cataracts would be delayed or prevented.

Although numerous apoptosis-inducing and -suppressing factors have been reported, apoptosis correlates closely with cellular growth or survival factors in many cases. Extracellular matrix, integrins, growth factors, and cytokines can induce or suppress apoptosis in mammary epithelial cells. Using transgenic mice expressing a dominant-negative form of the fibroblast growth factor receptor I in the lens, Chow et al. have found that fibroblast growth factor suppresses apoptosis of lens fiber cells. Li et al. have reported that calcimycin induces LECs apoptosis in organ cultures of the rat lens. Because in our study apoptosis was detected only among LECs that had differentiated into myofibroblast-like cells, myofibroblastic differentiation might correlate with apoptosis of LECs. We previously demonstrated that TGFβ increases the percentage of LECs expressing α-SMA, indicating myofibroblastic transdifferentiation, in culture within collagen gels. Moreover, TGFβ induces myofibroblastic transformation and apoptosis in cultured rat LECs. Because the aqueous humor contains a biologically significant amount (2.3 to 8.1 ng/ml) of TGFβ, approximately 60% being active form, TGFβ in the aqueous humor could induce myofibroblastic transdifferentiation and apoptosis in LECs after lens extraction. However, Ishizaki et al. have shown that apoptosis in cultured LECs is influenced by survival factors that are secreted by LECs themselves. These authors suggested that such factors act by an autocrine or paracrine mechanism. Although TGFβ seems the most likely inducer of apoptosis in vivo, further investigations are required to examine autocrine or paracrine secretion of apoptosis-inducing or -inhibiting factors by LECs, regulation of the receptors for these factors on LECs, and the effects of extracellular matrix or other microenvironmental factors on LECs.

Key Words
apoptosis, lens epithelial cells, secondary cataract, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, wound healing

Acknowledgments
The authors thank Takashi Kimura, Tatsushi Fujiwara, Kunio Fujita, and Shu Seki for their technical support and valuable advice and for the light and electron micrographs. The authors also thank Yoshihisa Oguchi and Akihiro Umezawa for their valuable suggestions on the manuscript.

References

21. Novotny GEK, Pau H. Myofibroblast-like cells in hu-


