Lens Epithelial Cell Apoptosis Appears to Be A Common Cellular Basis for Non-Congenital Cataract Development in Humans and Animals

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Abstract. Cataract is a major ocular disease that causes blindness in many developing countries of the world. It is well established that various factors such as oxidative stress, UV, and other toxic agents can induce both in vivo and in vitro cataract formation. However, a common cellular basis for this induction has not been previously recognized. The present study of lens epithelial cell viability suggests such a general mechanism. When lens epithelial cells from a group of 20 cataract patients 12 to 94 years old were analyzed by terminal deoxynucleotidyl transferase (TdT) labeling and DNA fragmentation assays, it was found that all of these patients had apoptotic epithelial cells ranging from 4.4 to 41.8%. By contrast, in eight normal human lenses of comparable age, very few apoptotic epithelial cells were observed. We suggest that cataract patients may have deficient defense systems against factors such as oxidative stress and UV at the onset of the disease. Such stress can trigger lens epithelial cell apoptosis that then may initiate cataract development. To test this hypothesis, it is also demonstrated here that hydrogen peroxide at concentrations previously found in some cataract patients induces both lens epithelial cell apoptosis and cortical opacity. Moreover, the temporal and spatial distribution of induced apoptotic lens epithelial cells precedes development of lens opacification. These results suggest that lens epithelial cell apoptosis may be a common cellular basis for initiation of noncongenital cataract formation.

CELL apoptosis has been described as programmed, in contrast to the accidental death of cells (Kerr et al., 1972). It either occurs as a normal physiological phenomena at certain stages during animal development (for review see Raff, 1992) or it can be triggered by a number of external signals such as hormone (Wyllie, 1980), γ-irradiation (Strasser et al., 1991), withdrawal of growth factors (Williams et al., 1990), and viral infection (Groux et al., 1992). Under normal physiological conditions, cell apoptosis helps to remove excess or unwanted cells or tissues such as vertebrate neurons (Cowan et al., 1984), and invertebrate and vertebrate larval tissues during metamorphosis (Kerr et al., 1974; Schwartz et al., 1993). In contrast, induced apoptosis can have serious pathological consequences (Carson and Ribeiro, 1993). For example, the bacterial pathogen *Shigella flexneri* can induce apoptotic death of macrophages, potentially leading to human dysentery (Zychlinsky et al., 1992). HIV-1 can induce apoptosis of $CD4^+$ T lymphocytes and presumably contribute to development of AIDS (Groux et al., 1992; Meyaard et al., 1992).

The lens of the vertebrate eye is a unique organ in that it is nonvascularized and noninnervated and contains only a single layer of epithelial cells on its anterior surface (Bloemendal, 1981). The epithelial cells remain quiescent in the central section, divide towards the equatorial area and terminally differentiate into fiber cells in the equatorial region (Papaconstantinou, 1967; Piatigorsky, 1981). This single layer of lens epithelial cells is essential for maintaining the metabolic homeostasis and transparency of the entire lens (Spector, 1991). Under normal physiological conditions, most of these cells have a relatively long life span. If such conditions are altered or disturbed by factors such as oxidative stress, the viability of the lens epithelial cells

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may be jeopardized, possibly resulting in opacification of the lens.

Previous studies have demonstrated that many different factors such as oxidative stress and UV can cause both in vivo and in vitro cataract formation (for reviews, see Harding and Crabbe, 1984; Bloemendal, 1992). However, it is not understood how these different factors induce cataractogenesis. To search for a common cellular mechanism, we have now studied the viability of the lens epithelial cells in normal and cataractous human lenses and normal and oxidatively stressed rat lenses. Surprisingly, all of the cataract patients examined have a substantial percentage of apoptotic lens epithelial cells while normal human lenses of comparable age have very few such cells. In rat lens organ culture, it is shown that hydrogen peroxide, an oxidative agent previously found to induce cataract formation (Garner et al., 1983; Giblin et al., 1987; Spector et al., 1993*a,b*), triggers epithelial cell apoptosis of the treated rat lenses that precedes temporally and spatially development of lens opacification. These observations suggest that lens epithelial cell apoptosis that is induced by various factors may be an early and critical event during cataract development.

Materials and Methods

Chemicals

In situ apoptosis detection kits were purchased from Oncor (Gaithersburg, MD). Radioactive compounds were obtained from Amersham Corp. (Arlington Heights, IL). Molecular biology reagents, various enzymes and DNA size markers were purchased from GIBCO BRL (Gaithersburg, MD), Stratagene (La Jolla, CA), New England Biolabs (Beverly, MA), and Promega Corp. (Madison, WI). The culture medium, hydrogen peroxide, and most other chemicals and antibiotics were purchased from Sigma Immunochemicals (St. Louis, MO).

Collection of Human Lens Capsule Epithelial Cell Samples

Cataracts were classified and capsule epithelial cell samples were collected during surgical operations by Physicians at Harkness Eye Institute. Normal human eyes were obtained from National Disease Research Interchange and the capsule lens epithelial cell samples were dissected at the laboratory. Each of the dissected capsule epithelial samples was transferred onto a sterile glass slide with a drop of physiological saline. The sample was then immediately divided into two parts: one for TdT labeling, the other frozen for DNA fragmentation assay.

Lens Organ Culture

The rats used in this investigation were handled in compliance with the "Guiding Principles in the Care and Use of Animals" (DHEW Publication, NIH 86-23). 4-wk-old Sprague-Dawley rats weighing ~100 g were killed by CO_2 inhalation. The eyes were removed and the lenses were carefully dissected by a posterior approach. Each of the dissected lenses was placed in a well of a 24-well culture plate containing 1.5 ml medium 199 (M-3769; Sigma) for ~1–2 h. Transparent lenses (without surgical damage) were selected for experimentation. The medium 199 for all rat lens experiments containing 26 mM $NaHCO_3$ as buffer was prepared with ion-exchange double-distilled water, sterilized by filtration through 0.22-mm filter (25942; Corning Inc., Corning, NY.) with a pH adjusted to 7.2 and an osmolarity of 300 ± 5 mosmols. To attempt to duplicate the possible in vivo pathological oxidative stress conditions present in some cataract patients (Spector and Garner, 1981), both nonconstant and constant H_2O_2 treatment were used. For nonconstant H_2O_2 treatment, each sample containing 3 transparent lenses was transferred into a 10-cm petri dish containing 30 ml medium 199, 150 μM H_2O_2, and then incubated at 37°C with a 5% CO_2 gas phase for the required time. The H_2O_2 concentration was assayed every hour and adjusted to 150 μM every 6 h (see Fig. 3 *A* for concentration change in a 6-h period). For constant H_2O_2 treatment, each sample containing three transparent lenses was transferred into a 6-cm petri dish containing 12 ml of medium 199 supplemented with 0.95 mM glucose, 60 mU (for the initial three 8-h period) or 50 mU (for the last three 8-h period) of glucose oxidase (G6125; Sigma) and 90 μM H_2O_2. In this condition, the concentration of hydrogen peroxide was maintained at 90 ± 5 μM for a period of 8 h, then the medium was changed and the same conditioned medium was restored for further culture.

In Situ Apoptosis Detection

In situ detection of lens epithelial cell apoptosis was carried out by modification of the procedures of Schmitz et al. (1991) and Gavrieli et al. (1992) using an Oncor detection kit (S7100). Lens capsule epithelial cell samples were dissected from normal human lenses, normal and hydrogen peroxide-treated rat lenses under a dissecting microscope and flat-mounted on glass slides. Cataract lens capsule epithelial cell samples were also flat-mounted. Each sample was then fixed with 500 μl 10% neutral buffered formalin followed by three changes of PBS wash. Later, the fixed samples were preincubated with 1× equilibration buffer (S7100-1; Oncor, Inc., Gaithersburg, MD) at room temperature for 10 min and then incubated with terminal deoxynucleotidyl transferase in the presence of digoxigenin-11 dUTP and dATP at 37°C for 60 min. The enzymatic reaction was terminated by incubating the samples in stopping buffer (S7100-4, Oncor) for 30 min. Then the samples were washed with 3 changes of PBS followed by 30-min incubation with anti-digoxigenin-peroxidase at room temperature. After this incubation, the samples were washed three times with PBS and allowed to react with 0.05% DAB (D5637; Sigma) followed by three washes with distilled water and then counter-stained with 0.5% methyl green (M-8884; Sigma). After washing with distilled water three times, the stained samples were dehydrated in butanol (Sigma), cleared in xylene (Sigma), and mounted with Permount (Fisher). After these procedures, the apoptotic cells in each sample were labeled brown or dark brown, while normal cells were stained light green.

DNA Fragmentation Assays

DNA fragmentation analyses were modified from Hogquist et al. (1991) and Prigent et al. (1993). Lens capsule epithelial cell samples from human normal or cataractous lenses and control or H_2O_2-treated rat lenses were combined into a microfuge tube (each combined sample contains portions of eight normal human lens epithelial cell capsule preparations, portions of 20 cataractous epithelial cell capsule preparations, or eight whole rat lens epithelial cell capsule preparations). 500 μl extraction buffer (10 mM Tris, pH 8.0; 10 mM EDTA, pH 8.0; 75 mM NaCl; 0.5% SDS and 150 μg/ml proteinase K) were added into each combined sample that was incubated at 50°C for 3 h. After incubation, the sample was microfuged for 20 min at room temperature. The supernatant was recovered for DNA precipitation by 2 vol of 100% ethanol (200 proof; Pharmaco Products Inc., Brookfield, CT) with 0.1 M NaCl. The precipitated DNA was washed with 70% ethanol and then treated with DNAse-free RNAse (18030-015; GIBCO BRL) for 60 min. Finally, the DNA sample was separated by 2.0% agarose gel electrophoresis and stained by ethidium bromide and photographed under UV illumination.

Electron Microscopy

Dissected rat lenses were cultured in 30 ml medium 199 with or without H_2O_2 for 24 h and the cultured rat lenses were fixed at room temperature in 2.5% glutaraldehyde prepared in 0.05 M sodium cacodylate, pH 7.2 (Kuszak et al., 1989). After buffer rinses, specimens were postfixed in 1% aqueous OsO_4 for 1 h. After additional buffer rinses, specimens were stained en bloc for 2 h at 60°C in 0.5% uranyl sulfate prepared in physiological saline. The whole fixed and osmicated lenses were then dehydrated through a graded series of ethanol and propylene oxide solutions followed by embedding in 100% Epon as reported previously (Brown et al., 1990). Using a Porter-Blum MT 2 microtome, sections (60–90 nm thick) were cut along the visual axis of each lens. The sections were retrieved onto uncoated 200-mesh copper grids and counterstained with lead citrate and uranyl acetate according to standard techniques. All grids were examined in a JEOL 1200 EX transmission electron microscope (TEM; Japan Electron Optics Ltd., Peabody, MA.) at 60 kV.

DNA Probe Preparation

Rat *c-fos* (Curran et al., 1987), mouse GAPDH cDNAs (Tso et al., 1985)

were amplified in bacterial strain DH 5α and purified by two continuous CsCl ultracentrifugations according to Ausubel et al. (1995). The cDNA inserts were recovered by double gel purification (Ausubel et al., 1995) and labeled with α[^{32}P]dATP (PB10204; Amersham Corp.) according to Feinberg and Vogelstein (1983).

RNA Preparation and Analyses

Total RNAs were extracted from the treated or untreated rat lenses according to Chomczyaski and Sacchi (1987) using a RNA buffer kit (CS-102; Biotecx Laboratories, Houston, TX). For Northern blots, 25 μg of total RNA was denatured, electrophoresed on formaldehyde-agarose (1.2%) gel and transferred to a supported nitrocellulose membrane (1465MC; GIBCO BRL) according to Thomas (1980). The RNA blot was then UV cross-linked for 5 min and baked at 80°C under vacuum for 2 h. Prehybridization was conducted according to Li and Riddiford (1994) at 42°C for 4–12 h in the following buffer: 50% formamide, 6× SSPE (0.9 M NaCl, 72 mM $NaPO_4$, pH 7.4, 7.2 mM EDTA), 5× Denhardt's solution (Denhardt, 1966), 1% SDS, 200 μg/ml denatured and sheared herring sperm DNA. Hybridization was conducted at the same temperature and buffer for 36–42 h with α-^{32}P-dATP–labeled specific probes at concentrations of 5×10^6 cpm/ml. After hybridization, the filter was washed once in 1 l of 1× SSPE, 0.2 % SDS for 30 min at room temperature, then once in 1 l of 0.1× SSPE, 0.1% SDS for 15 min at room temperature, and finally once in 1 l of 0.1× SSPE, 0.1% SDS at 60°C for 15 min, and exposed to Kodak XAR-5 film for 12–48 h. For reprobing, bound radioactive probes were removed from the previously-hybridized filter by washing twice with 10 mM Tris-EDTA buffer (pH 8.0) heated to 80°C.

Analyses of Nonprotein Thiol

Nonprotein thiol was determined by modification of the Ellman method (Ellman, 1958; Riddles et al., 1983). The lens was homogenized in 0.3 ml ice-cold acetate buffer (100 mM HAc, pH 2.0, 2 mM EDTA). 133 μl of the homogenate was mixed with 7 μl of ice-cold 100% trichloroacetic acid. The chilled preparation was centrifuged at 10,000 rpm for 10 min at 4°C. 40 μl of the aliquots were added to a tube containing 660 μl Tris-EDTA buffer (100 mM Tris-Cl, pH 8.2, 2 mM EDTA) and 35 μl 10 mM Dithiol-bis (2-nitrobenzoic acid). 2 min after mixing, the absorption at 412 nm was determined. The nonprotein thiol values were normalized by utilizing glutathione (GSH)[1] standards.

Measurement of Lens Wet Weight

The wet weight of pairs of rat lenses (one for control, one for experimentation) were weighed at varying times. The lenses were carefully removed from the culture plate and placed on a small piece of pre-weighed parafilm. Excess water was carefully removed with Whatman paper and then the lens was quickly weighed in an analytical balance (Mettler Instrument Inc., Hightstown, NJ).

Results

In Situ Detection of Apoptosis Reveals the Presence of a Significant Percentage of Apoptotic Cells in Human Cataractous Lenses

It is well known that cataractous lenses have damaged fiber cells (Garner et al., 1981; Harding and Crabbe, 1984). To determine the possible pathological involvement of lens epithelial cells during cataract formation, the viability of lens epithelial cells in human normal and cataractous lenses was examined. When samples of central epithelial cell capsule representing approximately one half of the section obtained from surgery (containing more than 50,000 epithelial cells per sample) were taken from 20 cataract patients 12–94 years old and analyzed by an in situ apoptosis detection procedure (Schmitz et al., 1991; Gavrieli et al., 1992), a significant percentage of the epithelial cells in these samples was found to be apoptotic (percentage was calculated by counting 2,500 cells in five random fields from each TdT-labeled sample, see Table I and Fig. 1, *b–d*). In contrast, TdT labeling of a whole mount representing one half of the entire capsule epithelium (~3.7–5 $\times 10^5$ cells) in each of eight normal human lenses revealed very few apoptotic cells (Table I and Fig. 1 *a*). Among the cataract patients, 10 had a mixture of both cortical and nuclear opacity, 4 nuclear, 4 cortical, 1 anterior subcapsular, and 1 posterior subcapsular opacity (Table I). The 12-yr-old patient with an anterior subcapsular cataract had an ice hockey injury to the eye that initiated the development of cataract, therefore excluding the possibility of congenital cataract.

DNA Fragmentation Assay Confirms Epithelial Cell Apoptosis in Human Cataractous Lenses

Nuclear chromatin fragmentation has been widely used as an index of cell apoptosis (Wyllie, 1980; Arends et al., 1990; Arends and Wyllie, 1991). During apoptosis, dying cells often have their DNA cleaved by cellular endonucleases in the internucleosomal regions (Arends et al., 1990; Arends and Wyllie, 1991), resulting in DNA fragments that are multimers or monomers of 180–200 bp

*Table I. Percentage of Apoptotic Cells in Normal and Cataractous Lenses**

Age	Sex	Type of Cataract	% Apoptotic Cells ± SD
35‡	M	None	<0.01
35‡	M	None	<0.01
74‡	F	None	<0.01
74‡	F	None	<0.01
81‡	M	None	<0.01
81‡	M	None	<0.01
86‡	F	None	<0.01
86‡	F	None	<0.01
12§	M	ASC	23.8 ± 2.8
44	M	N	9.7 ± 1.9
49	M	C and N	13.5 ± 1.1
52	F	C	16.2 ± 8.3
59	M	N	10.4 ± 5.5
62	M	C	31.8 ± 7.7
69	M	C and N	30.4 ± 2.6
70	M	C and N	28.5 ± 2.7
71	F	C and N	18.0 ± 4.9
72	F	C	16.3 ± 1.8
72	F	C and N	4.4 ± 1.9
75	F	C and N	19.8 ± 2.0
77	F	PSC	12.0 ± 3.1
78	F	C	33.6 ± 1.2
80	M	N	18.2 ± 3.2
83	F	C and N	41.8 ± 5.8
86	F	C and N	10.6 ± 3.6
86	M	C and N	7.9 ± 2.3
91	F	N	11.2 ± 0.1
94	F	C and N	19.2 ± 3.4

*Percentage was obtained by examining the epithelial cells in the TdT-labeled portion of each normal human lens epithelial capsule (~3.5–5 × 10^5 cells) and by counting 2,500 cells in five random fields of each capsule epithelial cell sample from the cataractous lenses. Statistical analyses was conducted according to Mendanhall (1983).
‡Normal eye lenses were obtained from National Disease Research Interchange.
§The cataract in this patient resulted from an ice hockey injury.
ASC, anterior subcapsular cataract; *C*, cortical cataract; *F*, female; *M*, male. *N*, nuclear cataract; *PSC*, posterior subcapsular cataract; *SD*, standard deviation.

1. *Abbreviations used in this paper*: GSH, glutathione; TdT, terminal deoxynucleotidyl transferase.

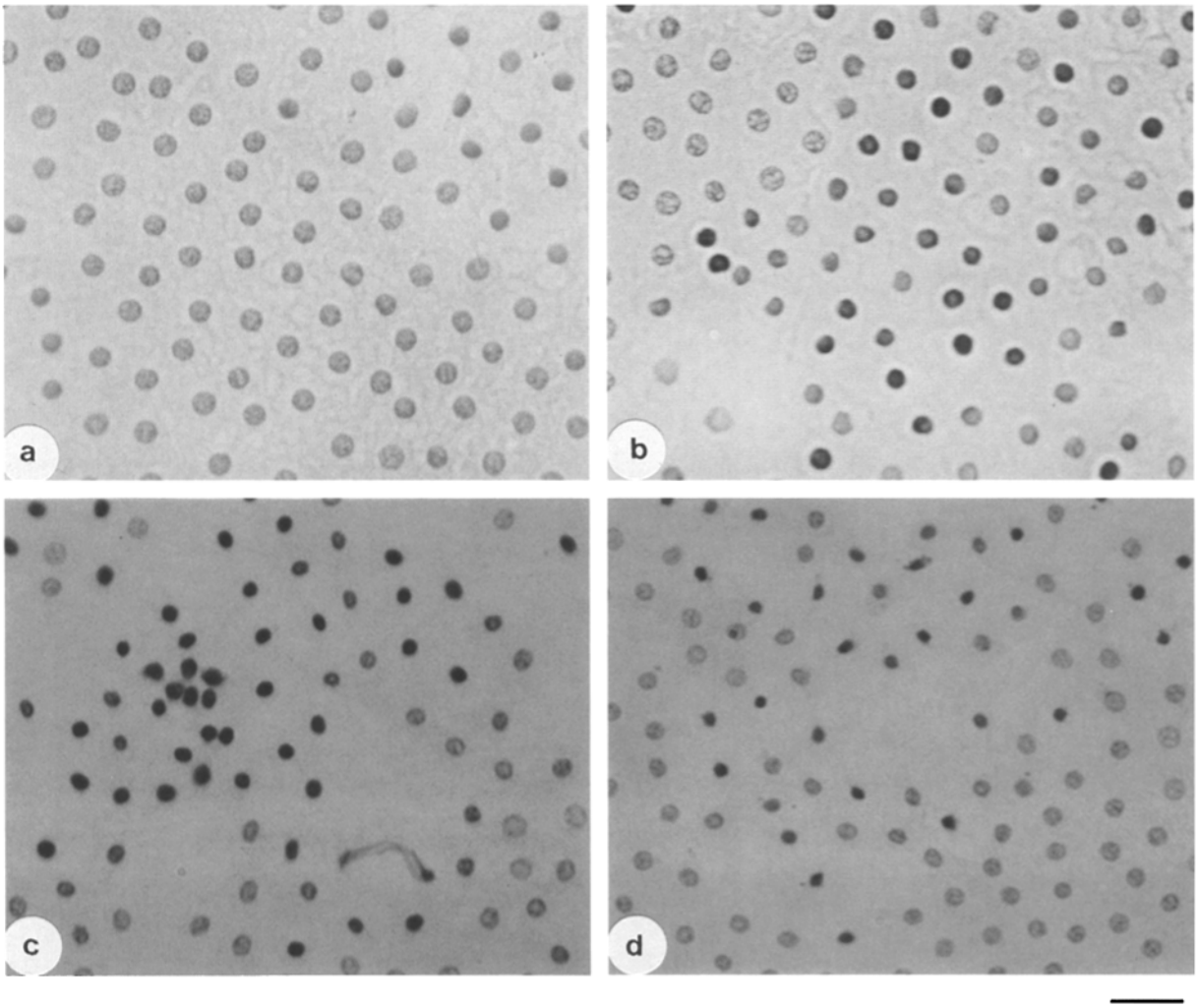

Figure 1. In situ detection of lens epithelial cell apoptosis in human normal and cataractous lens. Representative central capsule epithelial cell sections of normal human lens from the 81-yr-old male in Table I (*a*), lens with both cortical and nuclear cataract from the 69-yr-old male in Table I (*b*), lens with cortical cataract from the 78-yr-old female in Table I (*c*) and lens with nuclear cataract from the 80-yr-old male in Table I (*d*) were flat-mounted under a dissecting microscope, fixed with 10% neutral buffered formalin, and then labeled with TdT modified from previously described procedures (Schmitz et al., 1991; Gavrieli et al., 1992; see Materials and Methods) using an ApopTag kit (S7100; Oncor). Normal cells were counter-stained light-green, while apoptotic cells were labeled brown. Bar, 20 μm.

(Wyllie, 1980; Arends et al., 1990; Arends and Wyllie 1991). To further confirm that the TdT-labeled epithelial cells in human cataractous lenses are apoptotic, portions of the same lens capsule epithelial cell samples used for TdT labeling were combined and their genomic DNAs were extracted for fragmentation analyses (Hogquist et al., 1991; Prigent et al., 1993). As shown in Fig. 2, a typical DNA ladder of apoptosis was observed in the combined epithelial cell samples from the cataract patients (Fig. 2, lane *2*) but not from normal human samples (Fig. 2, lane *1*). These results confirm that the TdT-labeled lens epithelial cells from various kinds of noncongenital human cataract lenses were apoptotic.

Treatment of Rat Lenses with Varying Concentrations of Hydrogen Peroxide Causes Epithelial Cell Death

It has been well documented that animal cataract can be induced in vivo and in vitro by various factors (see Harding and Crabbe, 1984; Bloemendal, 1992 for reviews). Among these factors are oxidative stress (Garner et al., 1983; Giblin et al., 1987; Spector et al., 1993*a*,*b*), UV (Jose and Pits, 1985), and calcium treatment (Hightower and Reddy, 1982).

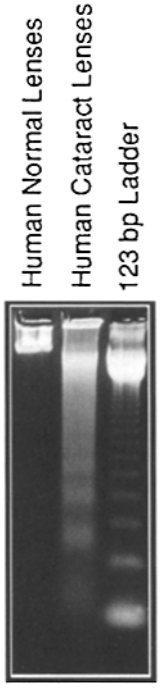

Figure 2. Typical DNA fragmentation assay of the lens epithelial cells from normal human lenses (*left lane*) or cataract lenses (*middle lane*). Lens capsule epithelial cell samples (portions of the same samples used for TdT labeling) were combined for extraction of genomic DNA as previously described (Hogquist et al., 1991; Prigent et al., 1993). The extracted DNAs were separated in 2.0% agarose gel, stained with ethidium bromide and photographed under UV illumination. 123-bp DNA ladder (GIBCO BRL) is shown in the right lane.

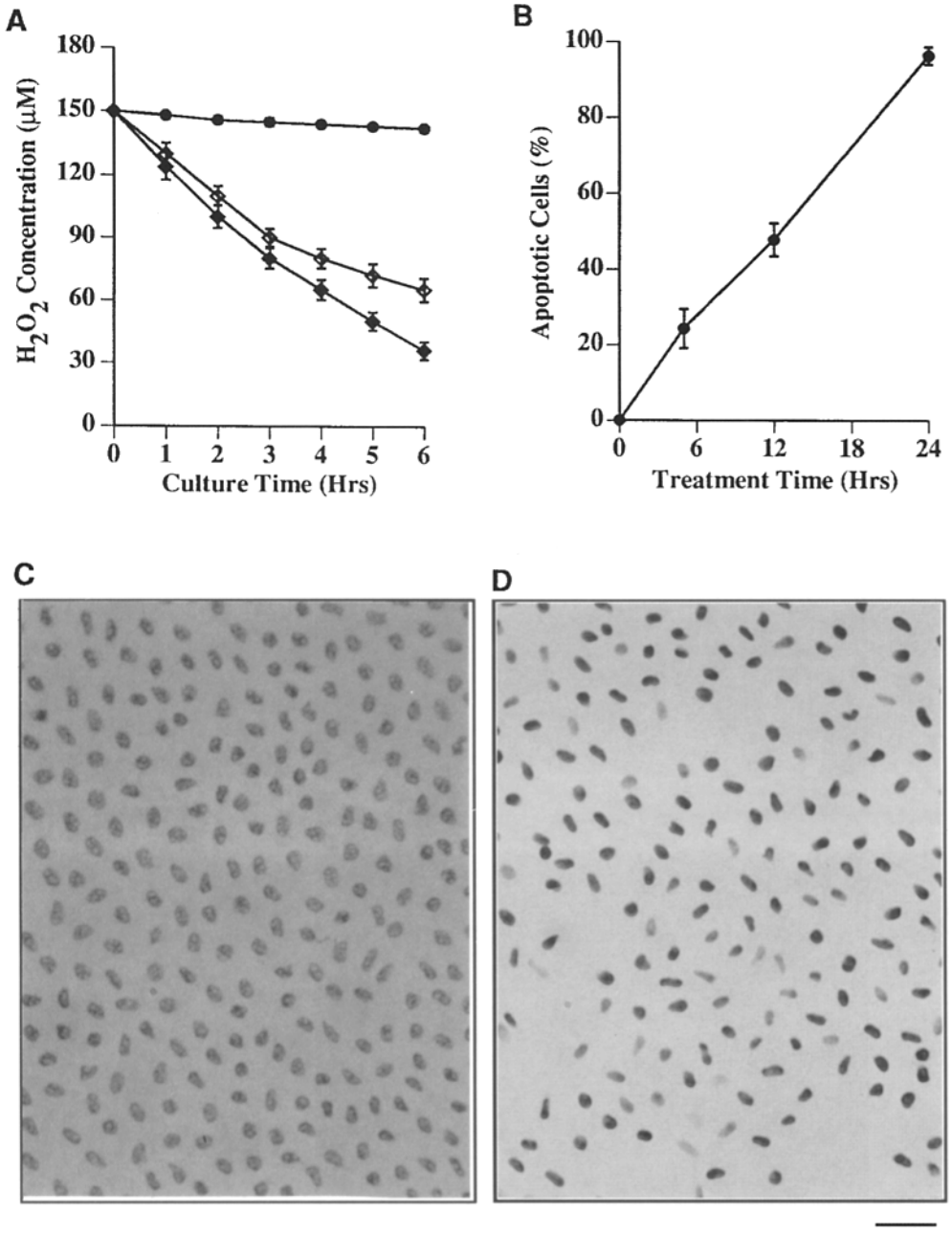

Figure 3. Nonconstant H_2O_2-induced lens epithelial cell apoptosis. (*A*) Change of H_2O_2 concentration in a 6-h period in medium 199 (-●-) or in medium 199 with four rat lenses (-◆- represents the first 6-h period of a 36-h incubation; -◇- the last 6-h period of the 36-h incubation. The H_2O_2 concentration was adjusted every 6–h). (*B*) Time-dependent curve of apoptosis induced by nonconstant H_2O_2. Rat lenses were incubated in medium 199 with H_2O_2 for varying lengths of time and then capsule lens epithelial cell sections were removed and processed for TdT labeling. The percentage of apoptotic cells was determined by counting 600 cells in four different fields (including both equatorial and central regions). The presented data is the average of three sets of experiments and the standard deviation is also shown. (*C* and *D*) In situ detection of lens epithelial cell apoptosis in control (*C*) or H_2O_2-treated rat lenses (*D*). Transparent rat lenses were incubated in medium 199 (*control*) or with nonconstant H_2O_2 for 24 h. Then the capsule epithelial cell samples from both control and treated lenses were flat-mounted, fixed with 10% neutral-buffered formalin and then labeled with TdT as described in Materials and Methods using an ApopTag kit (S7100; Oncor). Normal cells were counter-stained light-green, while apoptotic cells were labeled brown. Bar, 20 μm.

It is possible that the observed epithelial cell apoptosis in human cataract lenses may be triggered by these factors during their induction of cataract formation. To test this hypothesis, we first examined the effect of hydrogen peroxide (H_2O_2) at a nonconstant condition on cell viability and lens opacification in cultured rat lenses. Under such treatment (see Fig. 3 *A* for the change of H_2O_2 concentration within a period of 6 h), TdT labeling indicated that the number of epithelial cells undergoing apoptosis quickly rose as the treatment time increased (Fig. 3 *B*). After a 24-h treatment, almost all of the epithelial cells were apoptotic (Fig. 3, *B* and *D*). By contrast, the cells from rat lenses incubated for 24 h in the same medium but without H_2O_2 were rarely labeled by the TdT-procedure (Fig. 3 *C*).

H_2O_2-Treated Lens Epithelial Cells Display Characteristic Apoptotic Morphology

To confirm the apoptotic nature of the epithelial cells in the nonconstant H_2O_2-treated rat lenses, morphological changes of the lens epithelial cells were further examined under transmission electron microscope (TEM). As shown in Fig. 4, TEM examination of the central zone lens epithelial cells from lenses treated with nonconstant H_2O_2 for 24 h revealed ongoing apoptosis. As this process proceeded, the H_2O_2 treated cells shrank in size losing membrane contact with the overlying lens capsule, the underlying elongating fiber cells and from their epithelial cell neighbors (Figs. 4 *b* and 5, *c* and *d*). The nuclei of the apoptotic cells displayed condensed and fragmented chromatin (Figs. 4 *b* and 5 *d*) that was consistent with the results of nuclear chromatin fragmentation analyses (see below). Typical apoptotic morphology of cellular organelles, and apoptotic bodies were also observed (Fig. 4 *b* and 5, *c* and *d*). Thus, the morphological analysis of the treated lens epithelial cells suggested that H_2O_2 initiates lens epithelial cell apoptosis.

H_2O_2 Activates Endonucleases in Lens Epithelial Cells

To further confirm that treatment of rat lenses with nonconstant H_2O_2 triggers lens epithelial cell apoptosis, nu-

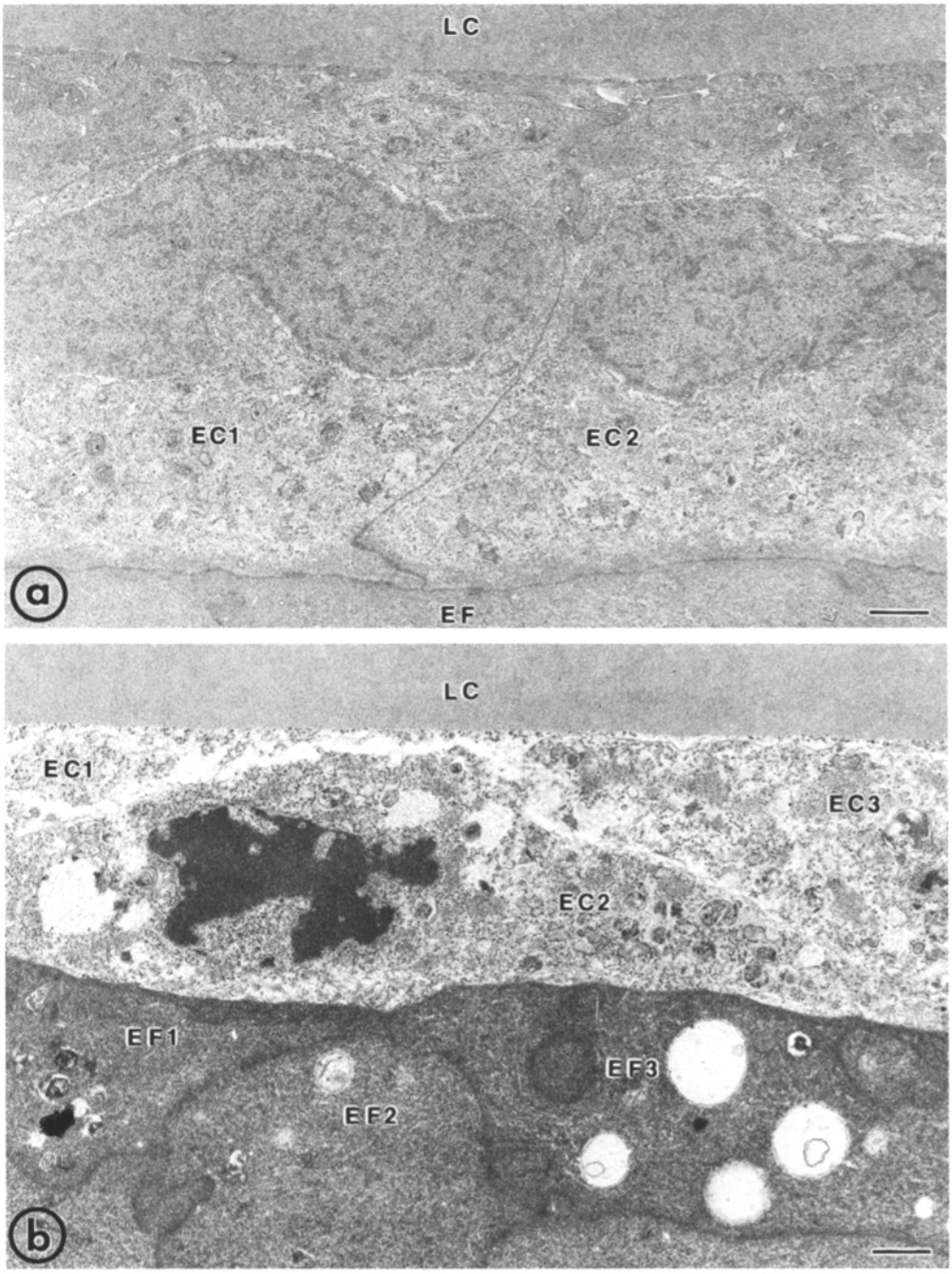

Figure 4. Low magnification comparison of control (*a*) and 24 h H_2O_2 treated (*b*) central zone epithelial cells (*ECs*). While the control epithelial cells have a normal appearance, the treated cells are markedly reduced in size. In this process, the membrane contacts between the lens capsule (*LC*) and the ECs basal membrane, the lateral membranes of ECs and the apical membranes of ECs and elongating fibers (*EFs*) are broken. The nuclei of the apoptotic treated cells also have condensed chromatin. Bars, 1.0 μm.

clear chromatin structure was analyzed. When rat lenses were treated with H_2O_2 for various times and the DNAs from these treated lenses were extracted and analyzed on the agarose gel, chromatin fragmentation could be detected after 5-h incubation (Fig. 6, lane *4*) and became definitive after 12 and 24 h (Fig. 6, lane *6* and *8*). In comparison with the 123-bp size marker (Fig. 6, lane *9*), it is apparent that the DNA banding patterns are indeed the results of monomers or multimonomers of 180–200 bp as observed in typical apoptosis (Arends et al., 1990).

H_2O_2 Induces Prolonged Expression of c-fos Gene

Utilizing a fos-lacZ transgenic mouse, Smeyne et al. (1993) demonstrated that the continuous expression of fos, beginning hours or days before the morphological demise of the cells, appeared to be an indicator of programmed cell death. When the total mRNA from rat lenses treated with nonconstant H_2O_2 were analyzed, the mRNA level for c-fos was clearly up-regulated from the first hour to 3 h (Fig. 7, lanes *2* and *3*). Then the c-fos mRNA level slightly decreased from 5 to 12 h (Fig. 7, lanes *4* and *5*) possibly due to negative autoregulation (Clark and Docherty, 1993). By 24 h, expression of the c-fos mRNA reached a second peak (Fig. 7, lane *6*) and was maintained during the next few hours (data not shown). The reference mRNA, GAPDH that has a much longer half life was not up-regulated (Fig. 7). This prolonged expression pattern of c-fos induced by hydrogen peroxide was found during treatment by calcimycin (Li et al., 1995) and UV (Li, W.-C. and A. Spector,

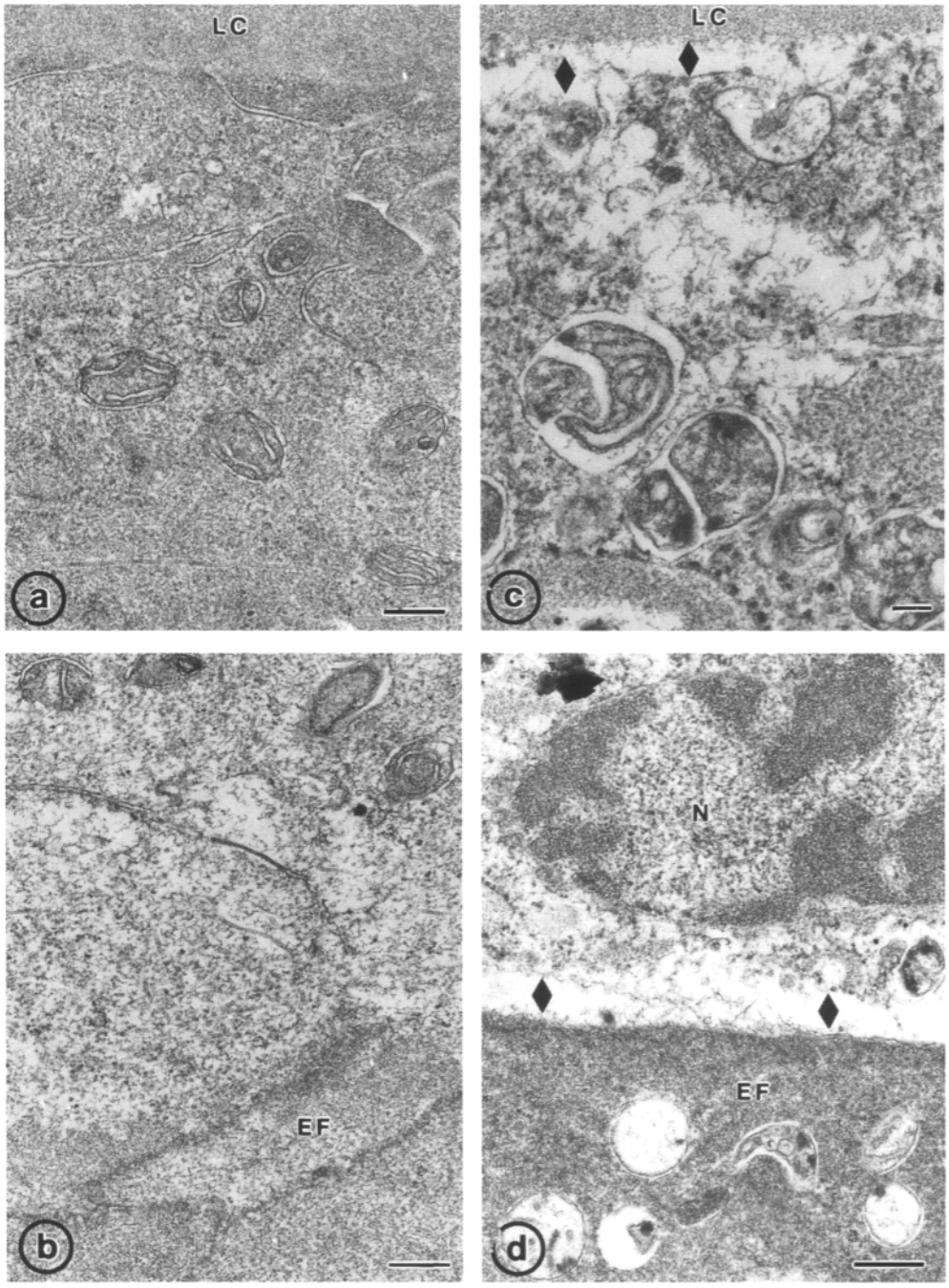

Figure 5. Intermediate magnification comparison of control (*a* and *b*) and 24 h H_2O_2 treated (*c* and *d*) central zone epithelial cells. The control cells show normal membrane contacts between basal membrane and LC, baso-lateral membrane (*a*), lateral membrane, apico-lateral membrane and apico-apical membrane of ECs and EFs (*b*). These cells also have typical numbers of organelles such as mitochondria, rough endoplasmic reticulum and cytoskeletal elements. In contrast, the treated cells (*c* and *d*) show separation (demarcated by black diamonds) of basal membrane and LC, basolateral membrane (*c*), lateral membrane, apico-lateral membrane, and apico-apical membrane of ECs and EFs (*d*). Membrane bound vesicles containing cellular fragments and intact organelles are seen in both the apoptotic and elongated fiber cells. Bars: (*a* and *b*) 0.25 μm; (*c*) 0.1 μm; (*d*) 0.5 μm.

unpublished results) and photochemical stress (Spector et al., 1995*a*,*b*) of cultured rat lenses. To our knowledge, c-fos is the only early responsive gene that shows such a prolonged expression pattern that parallels the apoptotic process. Since c-fos encodes a transcriptional factor (Curran and Vogt, 1993), it may be required for mediating changes in gene expression that leads to apoptosis.

H_2O_2-Induced Lens Epithelial Cell Apoptosis Precedes Cortical Cataract Development

As shown above, almost all of the lens epithelial cells became apoptotic after 24-h treatment with nonconstant H_2O_2 (Fig. 3, *B* and *D*). However, the complete cortical opacification did not develop till 36 h after treatment, while the control lens incubated in medium 199 alone remained transparent (data not shown). This result suggests that Lens epithelial cell apoptosis precedes cortical cataract formation. To further correlate the sequential and spatial changes of the lens transparency with apoptotic death of the lens epithelial cells, we developed a relatively constant H_2O_2 system in which H_2O_2 consumed by the cultured lenses was compensated by the generation of H_2O_2 by glucose oxidase in the presence of glucose. When rat lenses were treated with a constant concentration of 90 ± 5 μM H_2O_2 (approximately the average H_2O_2 concentration in the earlier nonconstant H_2O_2 experiments, see Fig. 3 *A*), the lens epithelial cells gradually became apoptotic.(Table

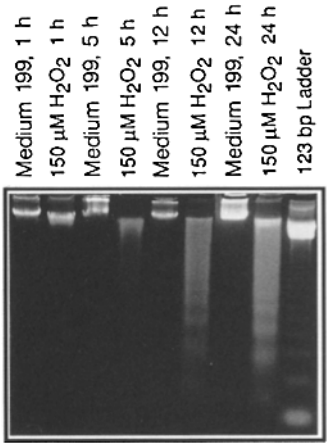

Figure 6. DNA fragmentation assays of the H_2O_2-treated lens epithelial cells. The dissected rat lenses were cultured in 30 ml 199 media containing nonconstant H_2O_2 for varying length of time. At the end of the incubation, the lens capsule epithelial cell samples from both control and treated rat lenses were dissected and placed into different precooled eppendorf tubes and then processed as described in Materials and Methods. The 123-bp DNA ladder is shown in the right side.

II and Fig. 8) similar to treatment with nonconstant H_2O_2 system. After a 3-h treatment, ~5% (central) to 12% (equatorial) of the lens epithelial cells were apoptotic (Table II and Fig. 8, *3Hr-E*), while this insulted lens was completely transparent (Fig. 8, *3Hr-E*). By 6 h after treatment, apoptotic lens epithelial cells have increased to 35% in the equatorial region and only 14% in the central area (Table II and Fig. 8, *6Hr-E*), and the development of the cortical opacity has started in the equatorial region (Fig. 8, *6Hr-E*). 18 h after treatment, 95% of the lens epithelial cells in the equatorial region and 70% of the cells in the central region were apoptotic (Table II and Fig. 8, *18Hr-E*). At this stage, the equatorial region was completely opaque, while the subequatorial and central regions still remained transparent (Fig. 8, *18Hr-E*) even though there is a very high percentage of apoptotic cells (Table II). By 24 h after treatment, all of the epithelial cells in the equatorial region and ~90% of the cells in the central area were dead through apoptosis (Table II and Fig. 8, *24Hr-E*). Now only the central region remains relatively transparent (Fig. 8, *24Hr-E*). In the next few hours, the remaining lens epithelial cells die via apoptosis and apoptotic cells become detached from the lens capsule, leading to loss of many cells in the TdT-labeled samples (Fig. 8, *36Hr-E*). The only area remaining transparent was the region around the visual axis (Fig. 8, *36Hr-E*) and this transparency was lost during the next 12 h (Fig. 8, *48Hr-E*). Thus, lens epithelial cell apoptosis clearly precedes development of lens opacification both temporally and spatially.

Other Changes Associated with Cataract Development Occur After Death of Most Lens Epithelial Cells

Previous work has shown that both a decrease of nonprotein thiol and an increase of wet weight in lenses subjected to oxidative stress were associated with development of lens opacification (Spector, 1991; Spector et al., 1993*a,b*). To distinguish these changes from lens epithelial cell apoptosis during cataract development, we also analyzed the temporal changes of nonprotein thiol and wet weight in both control and experimental lenses. As shown in Fig. 9, the dramatic changes of nonprotein thiol level and wet weight did not occur until most of the lens epithelial cells became apoptotic (18 h after the treatment by H_2O_2). Therefore, activated lens epithelial cell apoptosis is an earlier event compared with changes of these parameters and probably contributes to these later changes during oxidative stress induced cataract development.

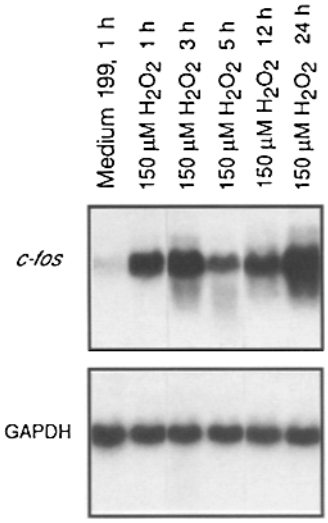

Figure 7. Northern blot analyses of H_2O_2 induced *c-fos* expression. 25 μg of total RNA was extracted from rat lenses exposed to nonconstant H_2O_2 for different times, denatured, separated on 1.2% formaldehyde-agarose gels, transferred to supported nitrocellulose membranes (GIBCO BRL), hybridized to *c-fos* or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes and washed under high stringency conditions as described in Materials and Methods. The *c-fos* mRNA in the controls from 3, 5, 12, and 24 h incubated lenses was undetectable under the same exposure condition and therefore is not shown.

Discussion

Lens Epithelial Cell Apoptosis Appears to Be a Common Cellular Basis for Cataract Development Induced by Widely Differing Agents

Decades of study on cataract development have shown that a wide variety of factors such as oxidative stress, radiation, and calcium treatment induce cataract formation (for reviews see Harding and Crabbe, 1984; Bloemendal, 1992). However, the common cellular basis underlying this induction by various factors has not been recognized.

Using in situ apoptosis detection and DNA fragmentation assay, the present paper demonstrates for the first time, that a significant percentage of lens epithelial cells die through apoptosis in all types of human cataract, indicating that apoptosis is associated with cataract formation. Of course, this association is not sufficient to establish apoptosis as a general cause for cataract development. Since it is difficult to obtain normal human materials, animal models have been used to further examine the relationship between apoptosis and cataractogenesis.

Two different H_2O_2 conditions were used in the in vitro rat lens organ culture to attempt to duplicate the possible in vivo pathological environment present in some cataract patients (Spector and Garner, 1981). Evidence from these in vitro experiments reveals that the observed lens epithelial cell apoptosis can be triggered by oxidative stress. Moreover, the in vitro experiments demonstrate that the induced lens epithelial cell apoptosis clearly precedes both temporal and spatial development of opacification, suggesting that lens epithelial cell apoptosis may be an initiating cellular event during cataract formation. This quick activation of lens epithelial cell apoptosis before development of lens opacification is also observed in calcimycin-

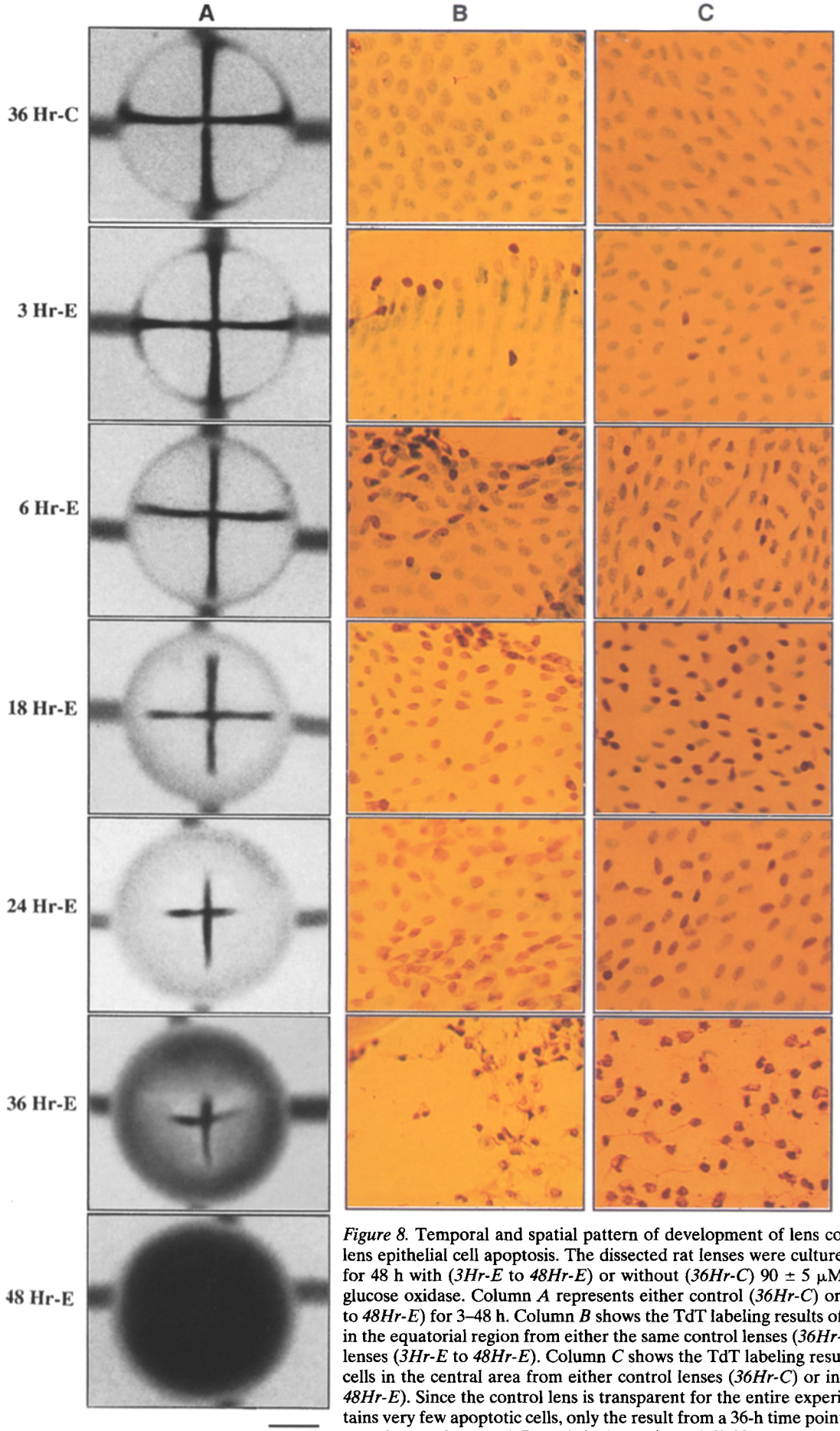

Figure 8. Temporal and spatial pattern of development of lens cortical opacification and lens epithelial cell apoptosis. The dissected rat lenses were cultured in 12 ml medium 199 for 48 h with (*3Hr-E* to *48Hr-E*) or without (*36Hr-C*) 90 ± 5 μM H_2O_2 maintained with glucose oxidase. Column *A* represents either control (*36Hr-C*) or insulted lenses (*3Hr-E* to *48Hr-E*) for 3–48 h. Column *B* shows the TdT labeling results of the lens epithelial cells in the equatorial region from either the same control lenses (*36Hr-C*) or the same insulted lenses (*3Hr-E* to *48Hr-E*). Column *C* shows the TdT labeling results of the lens epithelial cells in the central area from either control lenses (*36Hr-C*) or insulted lenses (*3Hr-E* to *48Hr-E*). Since the control lens is transparent for the entire experimental period and contains very few apoptotic cells, only the result from a 36-h time point is shown. *Hr,* hours; *E,* experiment; *C,* control. Bars: (*A*) 11 μm; (*B* and *C*) 20 μm.

*Table II. Time-dependent Apoptosis Induced by 90 ± 5 μM H_2O_2**

	3 H	6 H	12 H	18 H	24 H	36 H	48 H
				%			
Equatorial Region	12 ± 2	35 ± 3.5	65 ± 5.1	95 ± 2.5	100 ± 0	100 ± 0	100 ± 0
Central Region	5 ± 1.5	14 ± 2.5	37 ± 3.5	70 ± 5.1	90 ± 2.5	100 ± 0	100 ± 0
Total	8 ± 1.7	23.9 ± 3.1	46 ± 4.1	85 ± 4.5	94 ± 1.5	100 ± 0	100 ± 0

*Percentage of apoptotic cells were determined as described in Fig. 3.

treated (Li et al., 1995) and UV-irradiated rat lenses in the in vitro organ culture (Li, W.-C. and A. Spector, unpublished data). Thus, lens epithelial cell apoptosis is a common cellular event in H_2O_2, UV and calcimycin-induced cataract formation.

Diverse factors activating apoptosis are likely to exist in the ocular environment in the case of accident, disease, weak defense systems, increased environmental stress, or aging. Several lines of evidence suggest that death of the lens epithelial cells via apoptosis can lead to cataractogenesis. First, lens epithelial cell death will interrupt the lifelong growth of the human lens, therefore contributing to the thinness of cataract lenses (Goodman, 1964; Laursen and Fledilius, 1979) and the lower density of epithelial cells in the cataract lenses (Karim et al., 1987; Konofsky et al., 1987; Vasavada et al., 1991). Second, depletion of patches of lens epithelial cells will eliminate homeostatic epithelial cell control of the underlying fiber cells, leading to impairment of the integrity and transparency of these underlying fiber cells (Harding and Crabbe, 1984; Spector, 1991). For example, it is conceivable that the damaged lens epithelium will be leaky to calcium. The influx of calcium into the underlying fiber cells can activate the cellular cysteine proteinase calpains (David et al., 1989; Andersson et al., 1994) that then degrade cytoskeleton components (Yoshida et al., 1984*a*; Truscott et al., 1989) and lens crystallins (David and Shearer, 1984; Yoshida et al., 1984*b*). These processes eventually lead to crystallin aggregation (David et al., 1994) that together with other changes such as uptake of water and electrolytes leads to development of cortical and nuclear cataract (Shearer et al., 1992; Iwasaki et al., 1995). Thirdly, in the selenite-induced cataract, Hightower and McCready (1991) demonstrated that rabbit lenses whose anterior side was exposed to selenite displayed ionic imbalances and opacification of the entire lens, while lenses where only the posterior side was exposed remained transparent. A similar relationship was found in UVB irradiated rabbit lenses (Hightower and McCready, 1992). These results suggest that the lens epithelium is the first target in selenite and UV induced cataract. Finally, it is interesting to note that over-expressing viral gene E7 (Pan and Griep, 1994) or inactivating retinoblastoma tumor-suppressor gene *RB* (Morgenbesser et al., 1994) in transgenic mice lead to disruption of normal differentiation and apoptosis in the differentiating lens fiber cells. Accompanying this process is formation of deformed or cataract lenses.

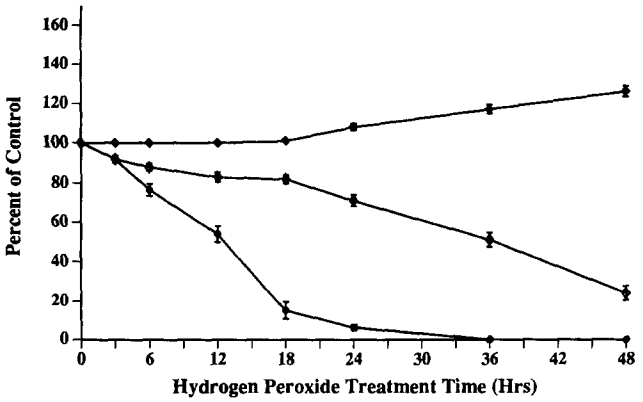

Figure 9. Variation of nonprotein thiol, wet weight, and viable cells with H_2O_2 treatment. Nonprotein thiol and wet weight were analyzed as described in the material and methods. Each point represents an average of two separate experiments after normalization against control values. -◆- Wet weight; -◇- NP-thiol; -●- viable cells.

Although in the present in vitro systems, H_2O_2 can cause, besides apoptosis, multiple other effects such as damage to DNA and membrane, drop of GSH and modifications of proteins, these multiple effects apparently occurred in a temporal sequence. In the photochemically stressed animal model where H_2O_2 is the major oxidant implicated in the formation of the cataract, Spector et al. (1995*a,b*) have recently demonstrated that lens epithelial cell modification probably initiated by a marked decrease in reducing capacity is the first event detected after stress is imposed. It appears to be immediately followed by DNA damage, inhibition of DNA synthesis, and membrane modification. These events precede and probably contribute to the death of the epithelial cells and occur within a few hours of initiation of the insult. All the other classical changes associated with cataract such as hydration, drop in GSH concentration, loss of ATP, lens protein modification, and opacification seemed to result from the initial changes in the epithelial cell layer after insult and occur in the post-insult period from ~1–12 d later. Similarly the early effects of H_2O_2 with the present conditions would be damaging to DNA (Spector et al., 1989; Kleiman et al., 1990) and membrane components (Fukui, 1976; Delamere et al., 1983; Giblin et al., 1987; Spector et al., 1993*a–c*) that probably leads to lens epithelial cell apoptosis, while other effects of H_2O_2 on the treated lenses such as GSH decrease, hydration, and lens protein modification, as demonstrated by our data and other studies (Fig. 9; McNamara and Augusteyn, 1983; Siezen et al., 1989; Spector et al., 1993*a–c*) becomes evident only at a much later time when most of the lens epithelial cells are dead (Fig. 9). Thus, it is apoptotic death of the lens epithelial cells that appears to initiate the cataract development.

In summary, our demonstration that lens epithelial cell apoptosis occurs in all types of human cataract examined and that lens epithelial cell apoptosis clearly precedes cataractogenesis with a number of different conditions sug-

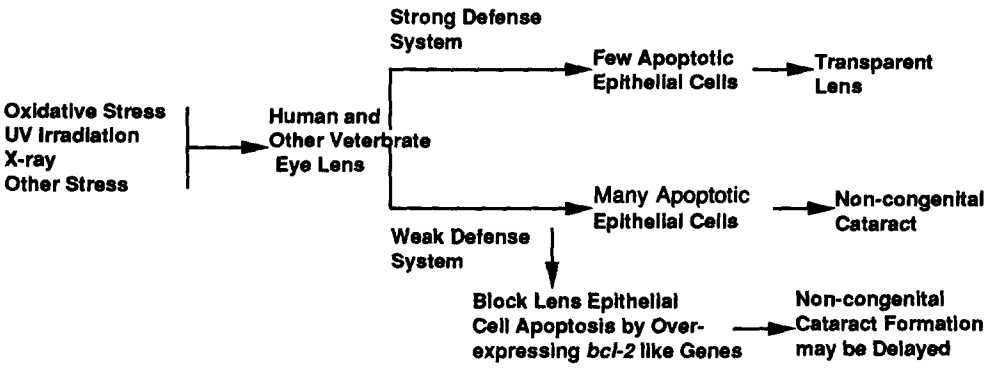

Figure 10. Model of induced lens epithelial cell apoptosis leading to noncongenital cataract formation. When the ocular lens faces environmental stress such as oxidative stress, UV, etc. it can detoxify such stress if a strong defense system exists and the level of insult is not overwhelming. However, when the defense system of the lens is weakened as in cataract patients, moderate environmental stress can trigger lens epithelial cell apoptosis that probably initiates human cataract. By over-expressing *bcl-2*–like genes in the lens to block epithelial cell apoptosis, cataract development may be delayed.

gests that induced lens epithelial cell apoptosis may be a common cellular basis by which different factors cause cataract formation (Fig. 10).

Comparison of Hydrogen Peroxide-Induced Apoptosis in Lens Organ Culture and Various Cell Lines

With in situ labeling, electron microscopy and DNA fragmentation assays, it is shown here that hydrogen peroxide induces epithelial cell apoptosis in the lens organ culture. The results with rat lens organ culture are consistent with a few previous studies where H_2O_2-induced apoptosis was observed in different cell lines (Lennon et al., 1991; Ueda and Shah, 1992; Hockenbery et al., 1993; Forrest et al., 1994). However, there is a clear difference between the conditions used in our organ culture and these different cell lines. Lennon et al. (1991) demonstrated that treatment of the U937 and HL-60 cells with 15 μM H_2O_2 for 12 h caused ~40% apoptosis. Forrest et al. (1994) showed that 10-min insult of mouse thymocytes with 0.5–10 μM H_2O_2 induced ~40% cell apoptosis after 1–6 h incubation in normal medium. In rat lens organ culture, experiments were also conducted with 25 μM H_2O_2. TdT labeling and DNA fragmentation assays all showed that treatment of rat lenses for 24 h with this low level of H_2O_2 (its concentration was adjusted every 2 h) did not induce apoptosis (data not presented). This is consistent with the fact that such a low level of H_2O_2 is normally present in vertebrate lenses and aqueous (for review see Spector, 1991) and therefore should not cause any pathological effect.

By contrast, Ueda and Shah (1992) reported that treatment of mouse renal tubular epithelial cells with 1 mM hydrogen peroxide for 2 h lead to apoptotic death of 50% of the treated cells. Hockenbery et al. (1993) showed that exposure of the pro B-lymphocyte to 1.0 mM hydrogen peroxide for 5 h induces apoptotic death of 40% of the treated cells. Even with a concentration as low as 250 μM H_2O_2, necrotic death of lens epithelial cells predominates in the treated rat lenses (data not shown). Such a high concentration of H_2O_2 was rarely found in human cataract (Spector and Garner, 1981).

How does H_2O_2 induce apoptosis? One possibility is the precipitous change of the cellular redox set point caused by H_2O_2. In the photochemically insulted rat lenses, the redox set point was rapidly shifted towards the oxidized form (Spector et al., 1995*a,b*). This cellular change in the redox set point exposes the cell to an oxidative environment, resulting in damage at the DNA level and a loss of membrane transport activity and upregulation of certain genes. It is conceivable that calcium influx will increase under these conditions, leading to an elevated intracellular calcium and thus trigger apoptosis. The calcium pump is very sensitive to H_2O_2-induced oxidation (Borchman et al., 1989). Alternatively, H_2O_2 may react with certain receptor like molecules. This interaction may activate certain signal transduction pathways, leading to apoptosis. The supportive evidence for this assumption is that the rapid induction of expression of the early responsive genes, *c-fos* and *c-jun*, by H_2O_2 requires involvement of both kinase and phosphatase activity (Li et al., 1994).

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