Apoptotic and Necrotic Mechanisms of Stress-Induced Human Lens Epithelial Cell Death

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Exposure to ultraviolet radiation (UVR) and reactive oxygen species (ROS) can damage the human lens and contribute to cataract formation. Recent evidence suggests that apoptosis in lens epithelial cells (LEC) is an initiating event in noncongenital cataract formation in humans and animals. The present study examines the cellular and molecular mechanisms by which environmental (ultraviolet B [UVB]) and chemical (hydrogen peroxide [H_2O_2], t-butyl hydroperoxide [TBHP]) stress induces cell death in an SV-40 immortalized human lens epithelial (HLE) cell line. Treatment of HLE cells with UVB, H_2O_2, and TBHP significantly decreased cell density with LD_{50} values of 350 J/m^2, 500 μM, and 200 μM, respectively. Cellular morphology, DNA fragmentation, and annexin/propidium iodide staining consistent with apoptosis was observed only in UVB-treated cells, whereas lactate dehydrogenase (LDH) release was significantly higher in H_2O_2- and TBHP-treated cells. In addition, activation of apoptotic stress-signaling proteins, including c-Jun NH2-terminal kinase (JNK), caspase-3, and DNA fragmentation factor 45 (DFF45) was observed only in UVB-treated cells. Inhibition of JNK activity increased UVB-induced cell death, suggesting that this pathway may serve a prosurvival role in HLE cells. These findings suggest UVB predominantly induces apoptosis in HLE cells, whereas H_2O_2 and TBHP induce necrosis. Exp Biol Med 229:1072–1080, 2004

Key words: ultraviolet radiation; human lens epithelial cells; apoptosis; necrosis; mitogen-activated protein kinases

The human lens epithelium comprises the most metabolically active cell layer of the lens and is the initial cell layer exposed to environmental and oxidative insult. Ultraviolet B (UVB) radiation, a biologically relevant source of environmental stress, can initiate deleterious changes in lens epithelial cells (LEC) including chromatin and nuclear condensation, inhibition of cell growth, and disruption of ionic homeostasis (1, 2). Both human and animal studies suggest that UVB exposure initiates the development of cortical and posterior subcapsular cataract (3–5).

The molecular basis of UVB damage has been linked to the generation of reactive oxygen species (ROS), although the exact basis of this damage is unknown (6). In addition to environmental stress, chemical stressors such as oxidant radicals derived from H_2O_2 or mitochondrial respiration may damage LEC (7). Elevated levels of H_2O_2 are reported in the aqueous humor of cataract patients (8) and can cause opacification of the lens *in vitro* (9). Hydrogen peroxide and t-butyl hydroperoxide (TBHP) have recently been found to deplete glutathione and damage ion pump activity in LEC (10). These changes can disrupt epithelial integrity and cause a loss of lens transparency.

Recent data suggest that LEC apoptosis is an initiating factor in noncongenital cataract formation (11, 12). LEC apoptosis occurs normally during embryological lens development (13), although its importance in the adult lens is not well-characterized. Dysregulation of LEC apoptosis, however, is associated with opacification of the rat lens and can be stimulated by both oxidative stress and UVB radiation (11, 12–14).

Recent evidence suggests that mitogen-activated protein kinases (MAPKs) are important in regulating lens apoptosis (15, 16) as well as cataractogenesis (17). Mitogen-activated protein kinases mediate signal transduction in response to mitogenic and environmental stress and regulate cellular events such as proliferation, differentiation, migration, and death (18–20). Three major MAPK cascades have been identified, including the extracellular signal-regulated kinase 44/42 cascade, which preferentially regulates cell growth and differentiation, and the c-Jun NH2-terminal kinase (JNK) and p38 cascades that mediate cellular stress responses. Lens epithelial cells possess all three major MAPK signaling cascades, of which JNK and p38 are strongly activated by ultraviolet radiation (UVR; Refs. 16, 21–24). Recently, the

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lens epithelium has been identified as the major site of the lenticular stress response, with activities of JNK and p38 highest in this cell layer when compared with other sections of the lens in human, bovine, and rat (25).

Ultraviolet radiation–induced apoptosis is regulated by a number of molecular processes, including the activation of cysteine-containing aspartate-specific proteases (caspases; Ref. 26). Caspase-3 is an effector caspase that, when cleaved, activates DFF45, a transcription factor involved in intranucleosomal DNA fragmentation and apoptosis (27, 28). Phosphorylation and activation of JNK is reported to occur prior to caspase-3 cleavage (29, 30). In addition, inhibition of caspase activity can block nuclear degeneration in differentiating LEC cultures (31) and suppress UVB-induced apoptosis of cultured human keratinocytes (32).

The present study examined the mechanism(s) by which environmental (UVB) and chemical (H_2O_2 and TBHP) stress induce human lens epithelial (HLE) cell death. We report that UVB predominantly induces apoptosis in HLE cells, whereas H_2O_2 and TBHP predominantly induce necrosis. This work lends support to the growing body of evidence that UVB exposure causes deleterious changes in the lens that may cumulatively lead to cataractogenesis.

Materials and Methods

Materials. Antibodies that recognize the active, phosphorylated form of JNK (designated P-JNK 54 and P-JNK 46) and c-Jun (designated P-c-Jun), as well as total JNK (JNK), total c-Jun (c-Jun), caspase-3 (pro and active forms), and cleaved DFF45, were purchased from Cell Signaling Technology (Beverly, MA). Hydrogen peroxide and TBHP were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture supplies were from Gibco Life Technologies (Valley Stream, NY). Annexin V–fluorescein isothiocyanate (FITC) Apoptosis Detection Kit was purchased from Oncogene Research Products (La Jolla, CA). Lactate dehydrogenase (LDH) cytotoxicity assay was purchased from Roche Applied Sciences (Indianapolis, IN). Suicide Track DNA ladder isolation kit and JNK inhibitor (SP600125) were from Calbiochem (La Jolla, CA).

Cell Culture. HLE cells (SRA 01-04, a SV-40 T-antigen transformed cell line) were provided by Dr. Venkat Reddy (Kellogg Eye Institute, University of Michigan, Ann Arbor, MI) and cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were incubated for 24 hrs prior to all experiments, unless otherwise noted. Cells were seeded and grown to 80% confluence prior to experimental procedures.

UVB Exposure and Oxidant Treatment. Ultraviolet B irradiation of HLE cells was performed using a 3UV transilluminator (UVP, Upland, CA). Prior to irradiation, growth medium was removed and tissue culture plates were inverted on a support at a fixed distance above the transilluminator. Following irradiation, FBS-containing growth medium was replaced and cells were further incubated for indicated times. Irradiance measurements were monitored with a UVX radiometer (UVP, Upland, CA) fitted with calibrated wavelength sensors.

Stock solutions of H_2O_2 (9.8 *M*) and TBHP (7.2 *M*) were prepared immediately before use in phosphate buffered saline (PBS) and administered for the appropriate times and concentrations.

Cell Density. Cell density was assessed using a crystal violet staining assay 24 hrs following incubation with UVB (0–1000 J/m^2), H_2O_2 (0–800 μ*M*), or TBHP (0–600 μ*M*; Ref. 33). Briefly, test medium was removed and 200 μl of crystal violet dye (0.2% in 2% ethanol) was added to each test well for 10 mins. Cells were washed with distilled water, and dye was reconstituted by SDS buffer (0.5% in 50% ethanol) and incubated at 37°C for 1 hr. Cell density was quantified by spectrophotometric detection at 610 nm. For time-course experiments, cells were treated with UVB (500 J/m^2), H_2O_2 (500 μ*M*), or TBHP (200 μ*M*) and stained with crystal violet 6, 12, 24, and 48 hrs posttreatment. Control cells receiving no treatment were included at each time point. Bromodeoxyuridine (BrdU assay) incorporation was also utilized to assess cell proliferation according to manufacturer's instructions (Oncogene Research Products, Cambridge, MA). All experiments assessing cell density were performed in triplicate with at least $n = 3$/experiment.

Annexin V/PI Staining. Quantification of stress-induced apoptotic and/or necrotic HLE was determined by FITC-conjugated annexin V and PI (Apoptosis Detection Kit; Oncogene) staining 4 hrs following treatment with UVB (500 J/m^2), H_2O_2 (500 μ*M*), or TBHP (200 μ*M*). After treatment, cells were washed in PBS and centrifuged at 200 *g* for 5 mins. Cellular pellets were resuspended in annexin V labeling solution and incubated according to manufacturer's instructions. Analysis was performed using an Olympus IX50 inverted fluorescent microscope fitted with appropriate filter cubes. A minimum of 300 cells were counted for each treatment group ($n = 4$).

Transmission Electron Microscopy. HLE cells were treated with UVB (400 J/m^2), H_2O_2 (400 μ*M*), or TBHP (300 μ*M*) and incubated for 12 hrs. After treatments, cells were prepared and transmission electron microscopy was performed as previously described (34).

Lactate Dehydrogenase Release Assay. Release of LDH from cellular cytosolic pools was quantified using the cytotoxicity detection kit (Roche Applied Sciences, Indianapolis, IN). Briefly, 96-well plates were seeded (2.0×10^4 cells per well) and allowed to incubate overnight at 37°C. Cells were treated with UVB (500 J/m^2), H_2O_2 (500 μ*M*), or TBHP (200 μ*M*) and incubated for 12 hrs. The supernatant was carefully removed, centrifuged, and transferred to a separate 96-well plate. Reaction mixture consisting of catalyst/dye combination was prepared and 100 μl was added directly to the cell supernatant. After

incubation at 15–25°C for 30 mins, absorbance was read using a spectrophotometer at 490 nm.

Analysis of DNA Fragmentation. Human lens epithelial cells were treated with UVB (400 J/m^2), H_2O_2 (500 μ*M*), or TBHP (200 μ*M*) and incubated for 6 or 24 hrs at 37°C. DNA fragmentation was performed using Suicide Track DNA ladder isolation kit (CalBiochem). Briefly, after appropriate treatments, cells (2×10^6) were harvested in extraction buffer, and the cell lysate was centrifuged at 15,000 *g* for 5 mins. The supernatant was carefully removed and prepared for DNA precipitation. After resuspension of the pellet, DNA was quantified and equal amounts (2 μg) were separated on an agarose gel (1.5%). DNA fragmentation was visualized under UV light after staining with ethidium bromide (5 μg/ml). All experiments assessing DNA fragmentation were performed in duplicate with $n = 3$ for each experiment.

Stress Signal Protein Immunoblotting. Activation of caspase-3 and DFF45 was assessed in HLE cells 6 hrs following treatment with UVB (500 J/m^2), H_2O_2 (500 μ*M*), or TBHP (200 μ*M*). Concentration-dependent activation of caspase-3 was determined 6 hrs following treatment with UVB (0–1200 J/m^2). Time-dependent activation of caspase-3 was determined 0–6 hrs following UVB (500 J/m^2) treatment. Treatment times were based on previous data demonstrating maximal DNA fragmentation and DFF45 cleavage 4–8 hrs following stress treatment (26). Activation of JNK was determined in HLE cells 30 min and 4 hrs following treatment ($n = 3$) with UVB (600 J/m^2), H_2O_2 (100 and 500 μ*M*), or TBHP (100 and 500 μ*M*). Treatment times were chosen based on previous data from this laboratory and others (17, 35). After appropriate treatments and incubations, cells were washed in ice-cold PBS (pH 7.4) and collected in cell lysis buffer. Crude proteins were extracted, quantified, and separated by electrophoresis (180 V, 1 hr) as previously described (16). After appropriate primary and secondary antibody incubations, protein signals were developed using chemiluminescence detection reagents (Bio-Rad, Hercules, CA). Membranes were exposed to Kodak X-OMAT AR film (Rochester, NY) for an appropriate length of time and developed according to manufacturer's recommendations.

Inhibition of JNK Activity. To inhibit JNK signaling, HLE cells were pretreated (1 hr) with the specific JNK inhibitor, SP600125 (0–50 μ*M*) followed by UVB (500 J/m^2) irradiation. Human lens epithelial cells were harvested either 30 min or 3 hrs following irradiation, and JNK and c-Jun activities, respectively, were determined by immunoblotting. To determine the effect of JNK inhibition on UVB-induced cell death, HLE cells were incubated (1 hr) in the presence or absence of SP600125 (50 μ*M*) followed by irradiation with UVB (0–600 J/m^2). Cell density was assessed 24 hrs following irradiation by crystal violet staining.

Data Analysis/Statistics. Data are expressed as mean ± SEM. Statistical differences between treatments were determined by analysis of variance (ANOVA) using SPSS (Chicago, IL) statistical analysis software ($P < 0.05$).

Results

Environmental and Chemical Stress Reduces HLE Cell Density. Treatment of HLE cells with UVB (0–1000 J/m^2), H_2O_2 (0–800 μ*M*), and TBHP (0–600 μ*M*) resulted in a decrease in cell density (Fig. 1A–C) as assessed by crystal violet staining. Treatment with 200, 400, and 600 J/m^2 UVB resulted in a 25%, 50%, and 70% reduction in cell density, respectively. Treatment with 200, 400, and 600 μ*M* H_2O_2 resulted in a 10%, 50%, and 80% reduction in cell density, respectively. Treatment with 200, 400, and 600 μ*M* TBHP resulted in a 10%, 80%, and 78% reduction in cell

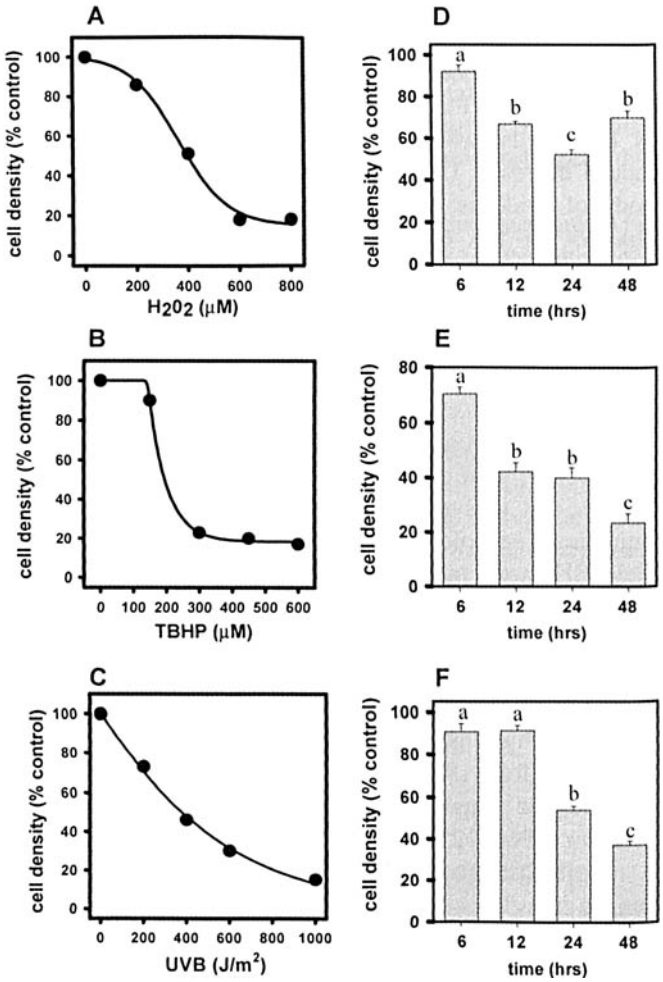

Figure 1. Concentration and time-dependent effects of environmental and chemical stress on cell density. Concentration dependence (left panel): human lens epithelial (HLE) cells were treated with H_2O_2 (0–800 μ*M*), t-butyl hydroperoxide (TBHP; 0–600 μ*M*), and ultraviolet B (UVB; 0–1000 J/m^2), and cell density was determined 24 hrs postexposure using crystal violet staining as described in *Materials and Methods.* Values expressed as percent survival of untreated controls (given as 100%). Time dependence (right panel): Cells were treated with H_2O_2 (500 μ*M*), TBHP (200 μ*M*), and UVB (500 J/m^2), and cell density was determined at 6, 12, 24, 48 hrs after exposure using the crystal violet assay. Values are expressed as percent control (given as 100%). Means without a common letter differ, $P < 0.05$.

density, respectively. Similar decreases in cell density were obtained using a BrdU cell proliferation assay (data not shown). Average dosages of stress required to reduce HLE cell proliferation by 50% (LD_{50}) obtained from crystal violet and BrdU assays for UVB, H_2O_2, and TBHP were 400J/m^2, 500 μ*M*, and 250 μ*M*, respectively. In order to allow for meaningful comparisons between the three stress treatments, concentrations that resulted in similar reductions in cell density were chosen for all subsequent experiments.

Stress-Induced Reductions in HLE Cell Density Are Time-Dependent. Figure 1D–F establishes the effect of time on HLE cell density after initial exposure to either UVB, H_2O_2, or TBHP. After 6, 12, 24, and 48 hrs, treatment with UVB (400 J/m^2) resulted in a 10%, 10%, 50%, and 70% decrease in cell density, respectively. After 6-, 12-, 24-, and 48-hr exposure, treatment with H_2O_2 (500 μ*M*) resulted in a 10%, 30%, 50%, and 35% decrease in cell density, respectively. After 6-, 12-, 24-, and 48-hr exposure, TBHP (200 μ*M*) resulted in a 30%, 60%, 55%, and 75% decrease in cell density, respectively.

UVB Increases Annexin V/PI Staining. Differentiation of necrotic cell death from apoptotic death was achieved via dual staining with annexin V-FITC and propidium iodide (PI) (Fig. 2). A significant increase in the percentage of cells (12.6%) staining positive for annexin V, compared with controls, was observed in HLE cells treated with UVB (400 J/m^2, $P < 0.05$). Hydrogen peroxide (400 μ*M*) and TBHP (300 μ*M*) treatment did not significantly increase annexin V staining. Treatment of HLE cells with UVB, H_2O_2, and TBHP significantly increased the percentage of cells staining positive for PI by 8.2%, 5.7%, and 17%, respectively, compared with controls ($P < 0.05$).

Stress-Induced Changes in Cell Morphology and Membrane Integrity. Representative electron micrographs of cells treated with UVB (500 J/m^2), H_2O_2 (500 μ*M*), and TBHP (200 μ*M*) are shown in Figure 3. Treatment with UVB resulted in morphological changes including cell shrinkage, membrane blebbing, and the formation of apoptotic bodies (Fig. 3B; Ref. 36). Treatment with H_2O_2 (Fig. 3C) and TBHP (Fig. 3D) resulted in cellular enlargement, vacuole formation, and organelle disorganization. Normal HLE cell morphology is shown in untreated controls (Fig. 3A; Ref. 37). Potential loss of membrane integrity associated with treatment of HLE cells with UVB, H_2O_2, and TBHP was monitored by LDH release. Treatment of HLE cells with H_2O_2 (500 μ*M*) and TBHP (200 μ*M*) for 6 hrs significantly increased LDH release by 70% and 130%, respectively, compared with those receiving UVB (500 J/m^2).

UVB-Induced DNA Fragmentation. DNA fragmentation is visualized as small, fragmented bands that create a characteristic laddering or smearing of DNA on ethidium bromide–stained agarose gels (38). Electrophoretic analysis of DNA for UVB (500 J/m^2), H_2O_2 (500 μ*M*), and TBHP (200 μ*M*) treated cells at both early (6 hrs) and late (24 hrs) times is shown in Figure 4. DNA fragmentation is evident only in cells treated with UVB (lanes 2 and 6) at both early and late times, with DNA fragmentation at 6 hrs more prominent than 24 hrs. DNA fragmentation was not evident at 6 or 24 hrs after treatment with TBHP (lanes 3 and 7) or H_2O_2 (lanes 4 and 8).

UVB Activates Caspase-3: Concentration and Time Dependence. To further characterize the mechanism(s) by which UVB induces apoptosis in HLE cells, concentration and time-dependent activation of caspase-3 was determined. Caspase-3 activation is marked by the cleavage of its precursor, procaspase-3. Cleavage of

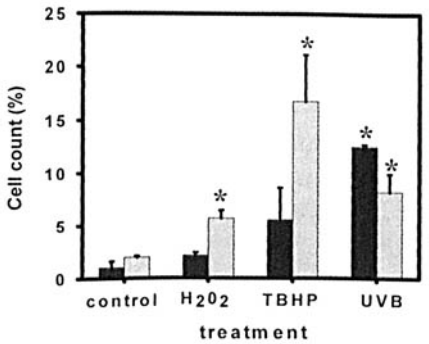

Figure 2. Ultraviolet B (UVB) increases annexin V/PI staining. Human lens epithelial (HLE) cells were treated with H_2O_2 (400 μ*M*), t-butyl hydroperoxide (TBHP; 300 μ*M*), or UVB (400 J/m^2) for 4 hrs followed by staining with annexin V and PI. Values are expressed as percent of annexin-positive (black bars) and PI-positive (gray bars) stained cells. A minimum of 300 cells were counted per group ($n = 3$). Data are expressed as mean ± SEM. Statistically significant increases in staining versus untreated controls is indicated (*$P <$ 0.05).

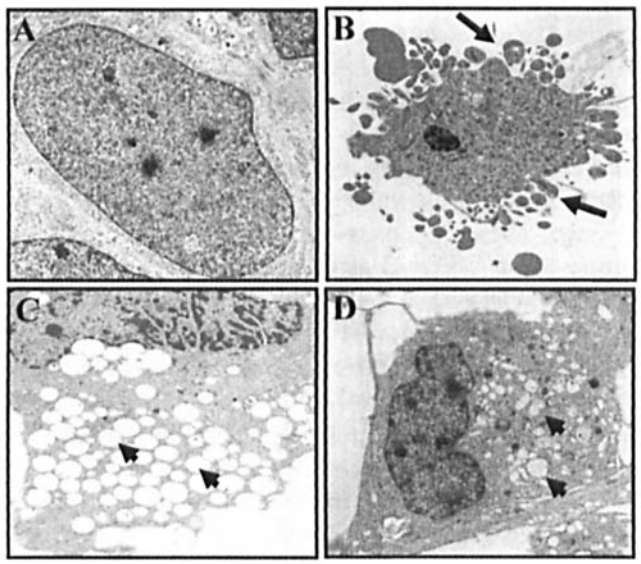

Figure 3. Stress-induced changes to human lens epithelial (HLE) cell morphology. Representative transmission electron micrographs of cell morphology 12 hrs after treatment with (A) control, (B) ultraviolet B (UVB; 400 J/m^2), (C) H_2O_2 (400 μ*M*), and (D) t-butyl hydroperoxide (TBHP; 300 μ*M*). Arrows, apoptotic bodies; arrowheads, vacuoles. Original magnification, ×7500.

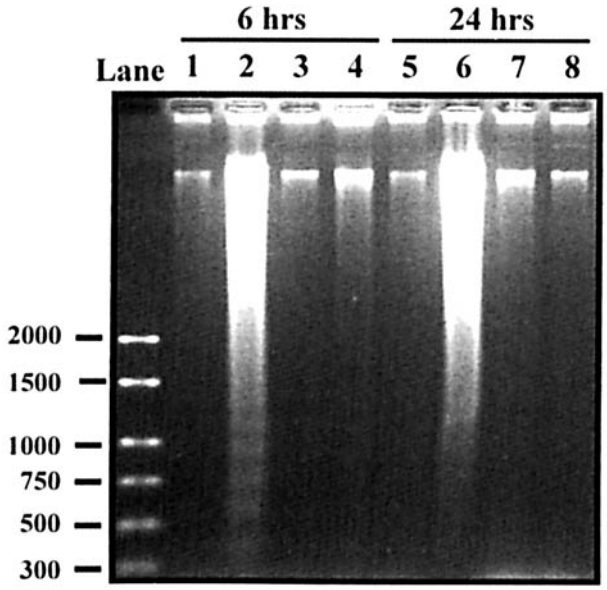

Figure 4. Ultraviolet B (UVB)–induced DNA fragmentation. Cells were treated with UVB (500 J/m^2, lanes 2, 6); H_2O_2 (500 μ*M*, lanes 4, 8); and t-butyl hydroperoxide (TBHP; 200 μ*M*, lanes 3, 7) for 6 hrs (lanes 1–4) and 24 hrs (lanes 5–8), and intranucleosomal DNA fragmentation was visualized by ethidium bromide staining after agarose gel electrophoresis. Standard DNA ladders and control cells (lanes 1, 5) are included for reference. DNA fragmentation patterns are representative of results obtained from at least three independent experiments.

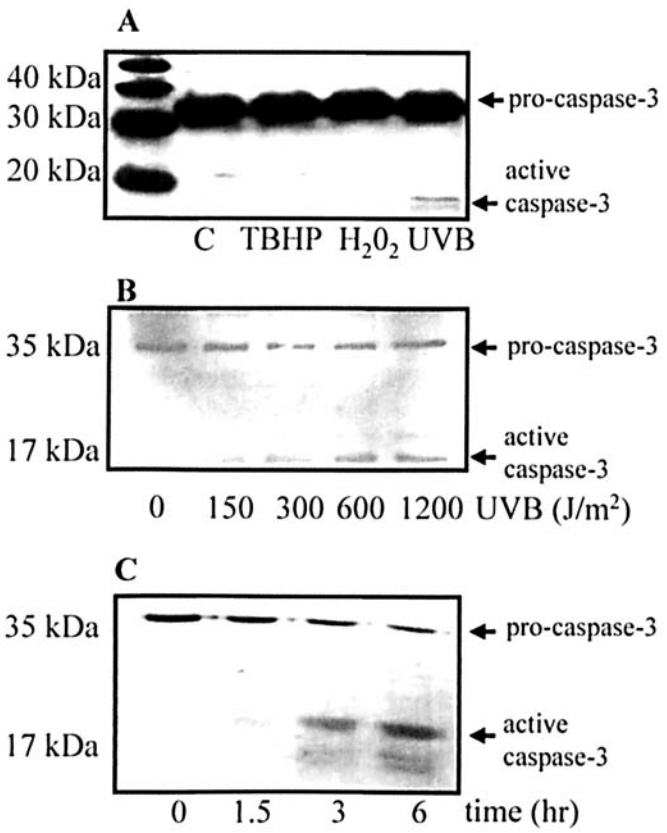

Figure 5. Stress-induced activation of caspase-3: Concentration and time dependency. Human lens epithelial (HLE) cells were treated with ultraviolet B (UVB; 500 J/m^2), H_2O_2 (500 μ*M*), or t-butyl hydroperoxide (TBHP; 200 μ*M*) harvested after 6 hrs and probed with caspase-3 antibody (A). Concentration-dependent activation of caspase-3, 6 hrs following treatment with UVB (0-1200 J/m^2) is given (B). Time-dependent activation of caspase-3, 1.5, 3, and 6 hrs following treatment with UVB (600 J/m^2) is given (C). Immunoblots are representative of results obtained from at least three independent experiments (n = 3/experiment).

procaspase-3 to activated caspase-3 was evident 6 hrs posttreatment with UVB (500 J/m^2; Fig. 5A), whereas neither H_2O_2 (500 μ*M*) nor TBHP (200 μ*M*) activated caspase-3. Stress-induced activation of DFF45, a downstream target of caspase-3, was also examined. Treatment with UVB (500 J/m^2) resulted in activation of DFF45, whereas neither H_2O_2 (500 μ*M*) nor TBHP (200 μ*M*) activated this protein in HLE cells (data not shown).

To further examine the effects of UVB on caspase-3 activation, concentration and time dependency was examined (Fig. 5B). Caspase-3 activation generally increased from (0–1200 J/m^2) with maximal activation at 600 J/m^2. Figure 5C shows the time-dependent changes in caspase-3 activation after UVB exposure (600 J/m^2). Activation of caspase-3 was observed as early as 1.5 hrs with maximal activation occurring at 6 hrs.

Stress-Induced JNK Activation and Role in UVB-Induced Cell Death. Stress-induced JNK activation in HLE at 30 min and 4 hrs is shown in Figure 6A. UVB treatment (600 J/m^2) induced JNK activation rapidly at 30 min, with decreased activation by 4 hrs. In contrast, H_2O_2 and TBHP (100 and 500 μ*M*) treated cells did not activate JNK at either time regardless of dosage. Total JNK protein levels remained constant for all test conditions.

SP600125 (SP), a specific JNK inhibitor, was used to inhibit JNK signaling in HLE cells. Pretreatment of cells (1 hr) with increasing concentrations of SP (2–50 μ*M*) result in decreasing JNK activation in response to UVB (Fig. 6B). Total JNK levels were independent of treatment. The effect of SP on UVB-induced c-Jun activation is given in Figure 6C. Treatment of HLE cells with UVB (500 J/m^2) increased c-Jun activity (p-c-Jun). Pretreatment of HLE cells with SP (50 μ*M*, 1hr) modestly decreased UVB-induced c-Jun activation. Treatment with SP alone did not alter c-Jun activation. Total levels of c-Jun did not change under these conditions.

The role of JNK inhibition on UVB-induced cell death was examined by pretreating cells with SP (0 or 50 μ*M*) 1 hr prior to exposure to UVB (0–600 J/m^2). Preliminary experiments with this compound indicate that it is not toxic to HLE cells at doses as high as 100 μ*M* (data not shown). Pretreatment with SP increased UVB-induced cell death by approximately 50%, 80%, and 40% at 100, 300, and 600 J/m^2, respectively, compared with those cells receiving UVB alone (Fig. 6D). Ultraviolet B–induced cell death was not altered by pretreatment with SB203580, a specific inhibitor of p38 activity (data not shown).

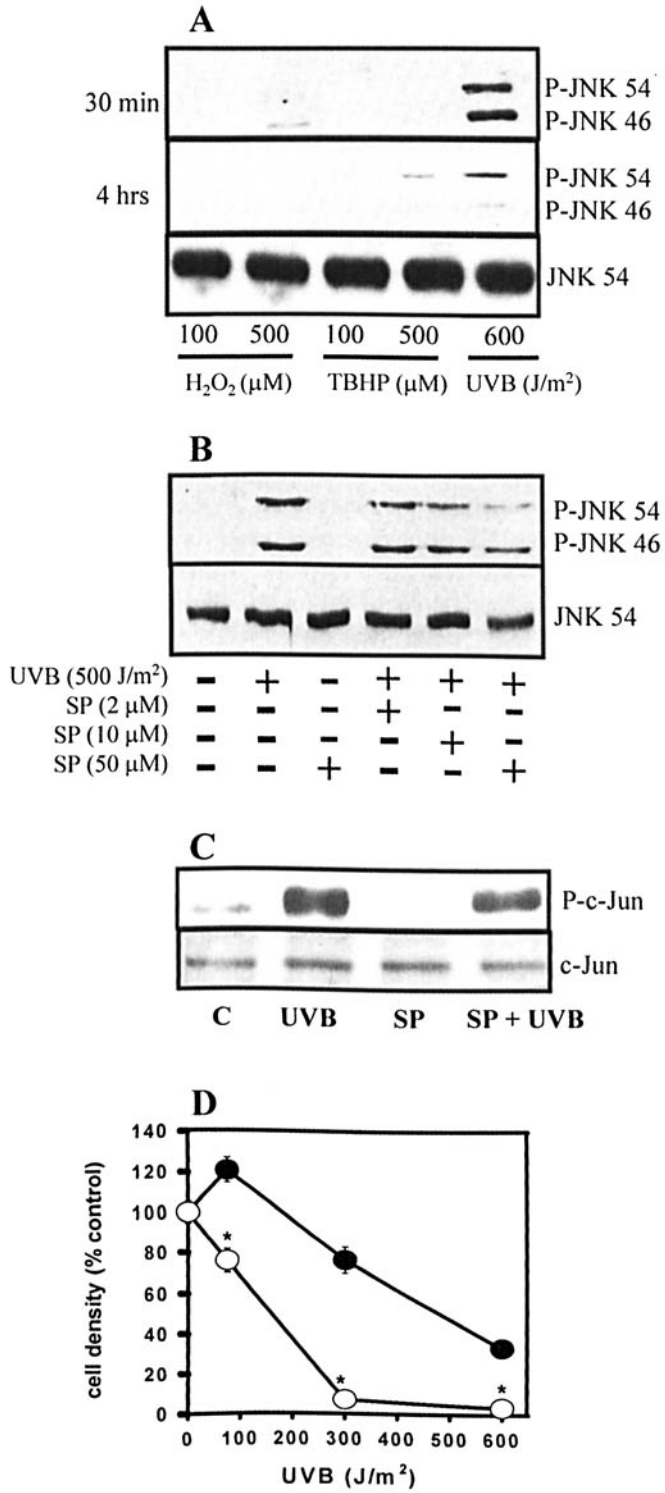

Figure 6. Stress-induced JNK activation and role in ultraviolet B (UVB)–induced cell death. (A) Human lens epithelial (HLE) cells were treated with either UVB, H_2O_2, or t-butyl hydroperoxide (TBHP); harvested after 30 min or 4 hrs; and probed with active (P-JNK 54/56) or total (JNK 54) c-Jun NH2-terminal kinase (JNK). (B) Human lens epithelial cells were pretreated (1 hr) in the absence or presence of the JNK inhibitor, SP600125 (SP), and harvested 30 min following UVB treatment. Active and total JNK was determined by immunoblotting. (C) Representative immunoblot ($n = 3$) of active c-Jun (P-c-Jun) and total c-Jun 3 hrs following treatment with UVB (500 J/m^2) or SP (50 μ*M*) alone and in combination. (D) Cell density (% control) in the presence (open circles) and absence (closed circles) of SP (50 μ*M*) 24 hrs following treatment with UVB (0–600 J/m^2) as assessed by crystal violet staining. Significant reductions in cell density between SP-treated (open circles) and untreated (closed circles) cells is given (*$P < 0.05$).

Discussion

The present study investigated the mechanisms by which environmental (UVB) and chemical (H_2O_2 and TBHP) stress induces HLE cell death. We present several lines of experimental evidence to suggest that UVB predominantly induces apoptosis in HLE cells, whereas H_2O_2 and TBHP treatment primarily result in necrosis. Although all three stressors induced HLE cell death, H_2O_2 and TBHP-induced cell death was much more rapid than that observed with UVB. Rapid cell death is associated with acute cellular injury characteristic of necrosis, whereas prolonged cell death is more characteristic of apoptosis. Hydrogen peroxide concentrations as high as 0.6 m*M* have been reported in the aqueous humor of the lens in persons with cataracts (8). This concentration compares with the LD_{50} of H_2O_2 used in this study.

Necrosis and apoptosis induce distinct morphological changes that clearly distinguish between these two modes of cell death. In this study, UVB treatment induced morphological changes (i.e., formation of apoptotic bodies) consistent with apoptosis, whereas H_2O_2- and TBHP-treated cells displayed morphological changes (i.e., formation of vacuoles) consistent with necrosis (39, 40). Our results contrast those of Wickert *et al.* (41), who report that UVB (1.5 J/cm^2) induces morphological evidence of necrosis in rat LEC. This discrepancy may be due to the higher UVR dose used in their study. Morphological observations in studies using a human lens model show that low doses of UVR (2 mJ/cm^2) induce apoptosis, whereas higher doses (10 mJ/cm^2) induce both apoptosis and necrosis (42). The observance in this study of some necrotic cell death in UVB-treated HLE cells is in agreement with this latter report.

In addition to morphological evidence, release of LDH from cytosolic pools is an indicator of loss of membrane integrity associated with necrotic cell death (43). Our results indicate that LDH release is significantly higher in those cells treated with chemical stressors compared with UVB. The modest increase in LDH activity observed in UVB-treated HLE cells may be due to a late apoptosis/necrosis stage of cell death.

DNA fragmentation is an early marker of apoptosis characterized by the presence of bands or "ladders" upon electrophoresis (44). In the present study, UVB treatment at both early and late timepoints induced DNA fragmentation patterns consistent with apoptosis, whereas treatment with H_2O_2 and TBHP did not result in DNA fragmentation, a finding consistent with necrosis (45). The decrease in UVB-induced DNA fragmentation observed at the 24-hr timepoint

may represent a late stage of apoptosis. Another early marker of apoptosis is the translocation of phosphatidylserine from the interior of the cell membrane to the exterior, detected by binding of FITC–labeled annexin V (46). This process is thought to occur prior to DNA fragmentation (45). In contrast, PI breaches leaky membranes, a characteristic event of late apoptosis and/or necrosis (47). In support of our morphological findings, UVB treatment increased annexin V staining relative to controls, suggesting apoptosis as the predominant mode of cell death. In contrast, H_2O_2 and TBHP treatment increased PI staining relative to controls, suggestive of necrosis as the predominant mode of cell death. Our study contrasts previous reports that indicate that H_2O_2 causes apoptotic cell death in rat LEC (11) and rat whole lens (48) models. This discrepancy may be due to differences in cellular models, the concentration of peroxide, or the time exposure used to treat cells. Choudhary *et al.* (49) report H_2O_2-induced apoptosis in cultured rat lenses at concentrations as high as 200 μM, with higher concentrations favoring necrosis. In contrast, we observed that lower concentrations of H_2O_2 did not induce apoptosis, and higher concentrations favored necrotic HLE cell death. Our finding that TBHP induced necrosis in HLE cells is consistent with observations of necrotic cell death in TBHP-treated PC12 cells (50).

The activation of MAPK signaling cascades by UVR has emerged as an important consideration in cataractogenesis (22, 51–52). Our results suggest that JNK activation is an early event in UVB-induced apoptosis in HLE cells and precedes the activation of caspase-3 and DFF45. The activation of JNK prior to UV-induced apoptosis is in agreement with others who show that JNK activation is required for UV-induced apoptosis in murine fibroblasts (53). Ultraviolet B–induced JNK activation preceded DNA fragmentation in HLE cells. This observation is in agreement with others who show that JNK activation precedes DNA fragmentation in UVC-treated Jurkat T-cells (54). Treatment with H_2O_2 and TBHP, at high and low concentrations, did not activate JNK or result in DNA fragmentation in HLE cells. This suggests that the response of HLE cells to UVB stress is distinct and utilizes existing stress-induced signaling pathways to execute cell death. Although UVR-induced JNK activation has been observed in numerous cell lines including keratinocytes (55–57), Jurkat T-cells (54), and rat PC-12 pheochromocytoma cells (58), its exact role in initiating apoptosis appears to be cell- and context-specific (59–61).

To further elucidate the mechanism by which UVB induced HLE cell death, the activation of two stress proteins associated with apoptosis, caspase-3, and DFF 45 was examined. These proteins mediate DNA fragmentation, and their activation is therefore critical to the apoptotic process (26). Caspase-3 cleavage has previously been shown to mediate JNK activation in human leukemia cells (62). We report an increase in activated/cleaved forms of caspase-3 and DFF45 in UVB-treated HLE cells. Furthermore, UVB-induced caspase-3 activation was dependent on both time and dose. In contrast, neither H_2O_2 nor TBHP activated caspase-3 or DFF45 in the present study. The importance of caspase-3 activation in apoptosis is supported by studies demonstrating that inhibition of this protein suppressed UVB-induced apoptosis in cultured human keratinocytes (32).

The induction of apoptosis post–UV exposure can be protective in nature, aimed at removing damaged cells and preventing neoplastic damage (63). Activation of JNK has recently been reported to be essential for the IL-3–mediated survival of FL5.12 cells (64). In order to explore the influence of UVB-induced JNK activation on HLE cell death, we investigated the response of HLE cells to the specific JNK inhibitor, SP600125. We report an increase in UVB-induced HLE cell death with JNK inhibition. This suggests that JNK activation in response to UVB may serve as a prosurvival (e.g., antiapoptotic) signal in HLE cells. Interestingly, activation of c-Jun, a downstream target of JNK, was only modestly reduced with SP600125. This suggests that the effects of SP600125 on UVB-induced cell death may be mediated by other JNK-activated transcription factors such as ATF-2, ELK-1, or p53 (65). It is of interest to note that inhibition of p38 activity did not alter UVB-induced HLE cell death (data not shown). This contrasts a recent report demonstrating the involvement of p38 activity, but not JNK, in cataract formation (16). The role of JNK and p38 signaling in UVB-induced lens apoptosis and cataractogenesis merits further consideration.

Data presented in this study establishes a preliminary sequence of cellular and molecular events leading to UVB-induced apoptosis in HLE cells. We report JNK activation as an early event in UVB-induced apoptosis, which occurs within 30 min after UVB irradiation. Subsequent activation of caspase-3 occurs as early as 1 hr and peaks 6 hrs post-UVB. Ultraviolet B–induced JNK activation occurs prior to morphological changes, nuclear DNA fragmentation, and annexin V staining and may serve as a prosurvival mechanism in HLE cells. Further clarification of the molecular events involved in HLE cell apoptosis may lead to the development of therapeutic strategies to prevent and/or delay cataractogenesis in both humans and animals.

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