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**UV-Induced Changes in Cell Cycle and Gene Expression
within Rabbit Lens Epithelial Cells**

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Duska Sidjanin,[†] David Grdina, and Gayle E. Woloschak[‡]

Center for Mechanistic Biology and Biotechnology
Argonne National Laboratory
9700 South Cass Avenue
Argonne, IL 60439-4833

[†]Department of Biological Sciences
Northern Illinois University
DeKalb, IL 60439-4833

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MASTER

[‡]To whom correspondence should be addressed. Phone: (708) 252-3312;
Fax: (708) 252-3387; e-mail address: Wolo@anlbem.bim.anl.gov

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SUMMARY

Damage to lens epithelial cells is a probable initiation process in cataract formation mediated by ultraviolet (UV) radiations. In these experiments, we investigated the effects of exposure to 254-nm radiation on cell cycle progression and gene expression in the rabbit lens epithelial cell line N/N1003A. RNA was harvested at various times following exposure to UV (254 nm) radiation and analyzed by dot blot and Northern blot hybridizations. These results revealed that during the first 6 h following exposure of the cells to UV, there was a dose-dependent decrease in accumulation of transcripts specific for histones H3 and H4 and an increase in the mRNAs encoding protein kinase C and β - and γ -actin. In this cell system, no changes in expression of *c-fos*, *c-jun*, α -tubulin, or vimentin transcripts were observed following UV exposure. Using flow cytometry, we detected an accumulation of cells in G1/S phase of the cell cycle 1 h following exposure to 254-nm radiation. The observed changes in gene expression, especially the decreased accumulation of histone transcripts reported here, may play a role in UV-induced inhibition of cell cycle progression.

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INTRODUCTION

The human eye is the only tissue in the body other than the skin that is directly exposed to UV and visible radiations (Lerman, 1980b). Epidemiological studies suggest that cataract formation is associated with long-term exposure to sunlight (Hiller, Giacometti and Yuen, 1977; Lerman, 1980a; Waxler and Hitchins, 1986). In vivo studies have shown that exposures of laboratory mice to UV radiation lead to the formation of cataracts (Zigman and Vaughan, 1974). Lens epithelial cells are thought to be a principal target for UVA-mediated and γ -radiation-mediated damage, yet very little is known about damaging effects of these agents on lens epithelium (Spector et al., 1989; Kleiman, Wang and Spector, 1990).

Exposure of mammalian cells to UV radiation triggers an alteration in the expression of a family (or families) of genes; this response is known as the UV-induced stress response (Herrlich, Ponta and Rahmsdorf, 1992; Ronai, Lambert and Weinstein, 1990). A similar cellular response has been observed with other stress-inducing agents such as ionizing radiation, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and hydrogen peroxide (Karin and Herrlich, 1989; Holbrook and Fornace, 1991; Woloschak and Chang-Liu, 1990a; Woloschak Liu and Shearin-Jones, 1990b). Immediate response genes are genes for which altered expression is evident within the first 4–6 h following exposure to any cellular signal. For radiation insults, these

immediate response genes code for proteins that are involved in cellular processes associated with radiation-mediated damage such as growth regulation, cell cycle arrest, differentiation, responses to tissue injury, inflammation, DNA repair, apoptosis, and others. The induction of genes such as *c-fos*, *c-myc*, and *c-jun*, as well as those for ornithine decarboxylase (ODC), p53, plasminogen activator, and stromelysin, may be mediated by multiple cellular processes (Herrlich *et al.*, 1992; Ronai *et al.*, 1990).

In this work we set out to examine UV-radiation-induced gene expression and cell cycle changes in lens epithelial cells that accompany exposure to UV radiation. Our experiments demonstrated changes in the expression of cell cycle genes (histones H3, H4, PKC, β - and γ -actin) concomitant with changes in cell cycle distribution.

MATERIALS AND METHODS

Cell culture. The rabbit lens epithelial cell line N/N1003A was generously provided by Dr. J. R. Reddan of Oakland University, Rochester, MI. This cell line was established from the lenses of newborn rabbits (Reddan *et al.*, 1980). The cells remain diploid in long-term culture, exhibit a normal chromosome pattern, and are nontumorigenic *in vivo*.

The cells were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 50 $\mu\text{g}/\text{ml}$ of gentamicin, 200 U/ml penicillin and 200 $\mu\text{g}/\text{ml}$ streptomycin (GIBCO, Gaithersburg, MD). The cultures were maintained at 36 °C in a water-saturated atmosphere containing 5% CO_2 (Reddan *et al.*, 1980).

Irradiation procedures. All irradiations were done using cell cultures that were 70% confluent. UV radiation (254 nm) was performed by using a GE30T8 30-W lamp. Prior to radiation exposures, cells were rinsed with Mg^{2+} -free, Ca^{2+} -free PBS. Cells were irradiated dry 55 cm away from the source and the UV dose rate was 2.5 W/m^2 . Cells were exposed for 2 s, 5 s, or 10 s and they received 5, 12.5, or 25 J/m^2 of 254-nm radiation. After irradiation, the cells were covered with complete medium and returned to incubators for 1, 3, or 6 h, after which they were harvested. Control cells were exposed to a dry environment for the same times as the irradiated cells. No effect on viability

(as measured by trypan blue dye exclusion) was observed for cells kept dry for up to 10 s.

Total RNA isolation. We followed the method of Woloschak and Chang-Liu (1990a) and Woloschak *et al.* (1990b). Briefly, cell monolayers were scraped off the dishes with a rubber policeman in 0.3 mL of 3 M guanidine isothiocyanate. Samples were phenol-extracted and precipitated overnight in ethanol at -20 °C. The pellets were recovered and dissolved in 3 M NaOAc (pH 6) overnight. Total RNA was stored as an ethanol precipitate at -20 °C.

DNA-excess dot blots. PolyA⁺ RNA was analyzed following the method described by Woloschak *et al.* (1986). Briefly, 0.5 µg of double-stranded, cloned, plasmid DNA was resuspended in 1 M ammonium acetate. The samples were dotted onto nitrocellulose filters using a dot-blot apparatus attached to a vacuum pump. The filters were washed in 3 × SSC (45 mM Na citrate, pH 7.4, 0.45 M NaCl), soaked for 1 h at room temperature in 1× Denhardt's solution (0.2% ficoll, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 3× SSC), and then baked in a vacuum oven at 80 °C for 2 h. Prior to hybridization, filters were washed for 2 h at 55 °C in hybridization buffer (50% formamide, 1× Denhardt's solution, 10 µg/mL Poly A, 50 µg/mL herring sperm DNA, 3× SSC).

PolyA⁺ mRNA was selected by applying total RNA on to oligo (dT) cellulose spun columns (Pharmacia-LKB, Piscataway, NJ) and quantitated by monitoring absorbance at 260 nm. Five micrograms of poly(A) mRNA (as

determined by A_{260}) was partially hydrolyzed with NaOH and neutralized with HCl. The mRNA was incubated at 37 °C for 45 min with 12 units of T4 polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 50 μ Ci of γ - 32 P-labeled ATP (3000 Ci/mmol, NEN, Boston, MA). Unincorporated 32 P-ATP was separated from RNA by Sephadex G25 column chromatography (Boehringer Mannheim Biochemicals, Indianapolis, IN) in 3 $\times$ SSC at room temperature. The labeled RNA was denatured by incubation at 90 °C for 1 min and cooled on ice for 5 min. The RNA was hybridized to nitrocellulose filters dotted with DNA probes at 50 °C overnight. Filters were washed three times for 1 h in 3 $\times$ SSC at 65 °C and three times 1 h each in 0.1 $\times$ SSC at 65 °C. Filters were dried and set up in the exposure cassettes over night. Storage phosphor plates were scanned on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) (Drmanac *et al.*, 1992).

Northern blots. Forty micrograms of total RNA was denatured in 50% formamide, 1.9 M formaldehyde, 0.2 M MOPS [3-(*N*-morpholino)-propane-sulfonic acid], 50 mM NaOAc, 1 mM Na₂EDTA, pH 7.5 for 15 min at 55 °C, and then was separated on 1.2% agarose gels in 0.2 M MOPS, 50 mM NaOAc, 1 mM Na₂EDTA, 2.2 M formaldehyde. The gels were stained with ethidium bromide and photographed under UV light. The RNA was transferred onto nitrocellulose filters by capillary action with 20 $\times$ SSC overnight. The filters were baked in a vacuum-oven at 80 °C for 2 h.

The cDNA probes were oligo-labeled with α - ^{32}P dCTP using a reaction labeling kit (3000 Ci/mmol, New England Nuclear, Boston, MA). Each labeled probe was hybridized to nitrocellulose filters, and hybridization conditions were 50% deionized formamide, 0.75 NaCl, 75 mM Na citrate, 25-50 mM Na phosphate, pH 6.5, 0.2% SDS, 0.2% bovine serum albumin, 0.2% herring sperm DNA at 43 °C. Prior to hybridization, all labeled probes were heat-denatured at 90 °C for 5 min and cooled on ice for 5 min. After hybridization, nonspecific binding was reduced by washing the blots at 43 °C three times for 30 min in 5 $\times$ SSC and then two times in 50% deionized formamide, 0.75 NaCl, 75 mM Na citrate, 25-50 mM Na phosphate, pH 6.5, 0.2% SDS, 0.2% bovine serum albumin, 0.2% herring sperm DNA. The blots were then dried and exposed to phosphorimager screens overnight and analyzed.

cDNA probes. Histone H3 and H4 probes were obtained from Dr. Stein (Carozzi et al., 1984; Sierra et al., 1982). Probes of c-jun, c-fos, and c-myc were obtained from the American Type Culture Collection. Protein kinase C (PKC) I, II, and III probes were obtained from the Genetics Institute (Knopf et al., 1986). β -Actin, γ -actin and p-actin probes were provided by Dr. Kedes (Gunning et al., 1983), and α -tubulin by Dr. Veneziale (Mayo Clinic).

Flow Cytometry. Cell monolayers that were 70% confluent were irradiated as described above and returned to incubators for 1, 3, or 6 h. The cells were removed from plates by trypsinization (1 $\times$ trypsin/EDTA, GIBCO),

washed twice with PBS, and resuspended in cold 70% ethanol in total volume of 1 mL. The DNA content was determined using protocol of Sigdestad et al. (1988). The cells were pelleted, ethanol was removed, and 0.5–1 mL of 2 $\mu\text{g/mL}$ of 4',6-diamidino-2-phenyl indole (DAPI; Sigma Chemicals, St. Louis, MO) was added to the cell pellet. The aliquot was analyzed by Partec Analyzing System (PAS-II, Germany) and Acqcyte computer program (Phoenix Flow Systems, San Diego, CA).

RESULTS

Effects of UV on specific gene expression. PolyA⁺ RNA from irradiated and control cells was 5'-labeled and hybridized to nitrocellulose-immobilized DNA clones. The filters were analyzed using a PhosphorImager to determine the relative levels of gene-specific mRNA at 1, 3, and 6 h following irradiation. Control mRNA was derived from cells that did not receive any radiation but were exposed to the conditions of the radiation exposure. In initial experiments, we screened 28 clones for changes in expression following radiation exposure. Of the 28 clones, only four showed induction following UV exposure. Those clones showing no change in lens epithelial cells following UV exposure were *c-fos*, *c-jun*, *c-myc*, α -tubulin, vimentin, and others. The results also showed an increase in expression of mRNA specific for PKC-I and PKC-II, but there was no change in PKC-III mRNA transcript accumulation (Fig. 1). We also detected an increase in the levels of mRNA for β - and γ -actin and no change in the levels of α -tubulin and vimentin transcripts (Fig. 2).

For analysis of histone transcripts, total cellular RNA (rather than polyA⁺ RNA) was used since histone RNA has no polyA tails. Total RNA samples analyzed by Northern blots were hybridized to the indicated cDNA clones (Fig. 3). The levels of RNA specific for histones H3 and H4 decreased with time as a consequence of UV exposure (Fig. 3). Northern blots also revealed an increasing accumulation of PKC-I (β -PKC) transcripts as early as 1

h that reached maximum levels 3 h following UV irradiation. Northern blots confirmed that there was no change in RNA levels for *c-jun* after exposure to 12.5 J/m^2 but did establish the induction of γ -actin mRNA during this time frame (Fig. 3).

We next wanted to determine if decreased amounts of the H3 histone RNA was related to the dose of UV radiation that cells had received. Cells were exposed to 5 or 25 J/m^2 of UV and RNA was collected at 1, 3, and 6 h after exposure. Figure 4 shows that the decrease in accumulation of H3 histone RNA was more pronounced with greater doses of UV. However, 6 h after exposure to 5 J/m^2 there was a recovery of the RNA to control levels. For cells that received 25 J/m^2 , there was a significant decrease of the H3 histone transcript as early as 1 h following exposure (Fig. 4).

UV-induced cell cycle effects. The effects of UV radiation on progression of the lens epithelial cells through the cell cycle were investigated. Cells were exposed to different doses of UV (5, 10, or 25 J/m^2) and collected after different incubation times (1, 3 or 6 h). The controls were unirradiated cells harvested at each of the three different time points plus time zero. There was no significant difference among the four controls (not shown). The progression of cells through the cell cycle was measured by labeling DNA with DAPI and analyzing DNA profiles by flow cytometry. Cells that received 5 J/m^2 showed a significant decrease in G2 cells relative to unirradiated controls, indicating a cell cycle arrest at the G1/S checkpoint (Fig. 5). This decrease of cells in G2

was more pronounced as cells received greater doses of UV (Fig. 5). When cells received 25 J/m^2 it appeared that the fraction of cells having G1 and S DNA contents increased as the fraction of cells in the G2 compartment decreased. Table I summarizes the effects of UV on cell cycle in these cells.

DISCUSSION

PKC is an enzyme that phosphorylates proteins at serine or threonine residues in the presence of Ca^{2+} and membrane phospholipids (Inoue *et al.*, 1977; Nishizuka, 1984). Induction of PKC by UV radiation was reported previously in other cell systems (Matsui and DeLeo, 1990; Peak, Woloschak and Peak, 1991). In our study with lens epithelial cells, induction of PKC mRNA was evident as early as 1 h and reached maximum levels 3 h following radiation exposure. It has been postulated that PKC plays a central role in cell signal transduction that ultimately leads to induction of stress response genes (Herrlich *et al.*, 1992).

There is some evidence showing that PKC plays a central role in the induction of stress-induced immediate response genes such as *c-jun*, *c-fos* and *egr-1* (Woloschak *et al.*, 1990b; Fornace, 1992; Hallahan *et al.*, 1991). The increase in expression of *c-jun* and *c-fos* cellular oncogenes after exposure to DNA-damaging agents has been postulated to play an important role in the induction of cellular defense mechanisms (Ronai *et al.*, 1990). However, we did not observe any change in levels of mRNA specific for *c-jun* as measured by DNA-excess dot-blots and northern blotting or for *c-fos* and *c-myc* as measured by dot blots using times, doses, and conditions reported by others to induce these genes (Herrlich *et al.*, 1992; Hollander and Fornace, 1989; Woloschak *et al.*, 1986). It is possible either that lens epithelial cells require

different conditions for induction of these genes or that genes are not induced in lens cells following UV stress. The reasons for this are unknown, but could be related to the absence of repair of UV-induced DNA strand breaks in lens cells (Sidjanin and Peak, unpublished information). Experiments are underway to test this.

We observed a change in the levels of γ -actin and β -actin transcripts following exposure to UV. Previous work by our group has documented decreased amounts of β -actin mRNA and increased accumulation of γ -actin mRNA following exposure to γ rays (Woloschak and Chang-Liu, 1991). The functional significance of these changes is unknown. Zigman (1992) reported that UV induces a breakdown of filamentous actin in the cells and the presence of clumped actin near the cell nucleus in N/N1003A cells. This suggests that disruption of the cytoskeleton by UV radiation may be associated with the increased levels of γ -actin mRNA. The results from this study showed that UV radiation modifies the expression of some histone transcripts. Some studies suggest that histone RNA stability is associated with the cytoskeleton; Zambetti, Stein and Stein (1987) determined that histone H4 RNA was preferentially found in nonmembrane-bound polysomes associated with the cytoskeleton.

In mammalian cells, the mRNA levels of nucleosome histones (H1, 2HA, 2HB, H3 and H4) increase and decrease 30- to 50-fold as the cells traverse S phase. These replication-dependent histone genes have unique characteristics:

1) they do not contain introns; 2) they are clustered in the genome and coordinately expressed; and 3) their transcripts lack poly(A) tails, though the 3' ends contain conserved stem-loop structures (Marzluff and Pandey, 1988). In northern blot analyses, we used total cellular RNA and observed that the amounts of histone H3 and H4 transcripts were decreased by exposure to UV radiation. As the cell progressed from G₁ to S phase, histone gene transcription is reported to change only three- to fivefold. Histone transcripts are considered to be regulated at the posttranscriptional level; this process is postulated to involve a nuclease having enzymatic activity specific for the histone transcript and coupled to DNA synthesis (Marzluff and Pandey, 1988). Little is known about the actual signal that links DNA synthesis to levels of histone mRNA. It has been postulated that histone expression and DNA replication may be regulated by the same mechanism (Graves and Marzluff, 1984). Han *et al.* (1987) showed that DNA synthesis is a requirement for expression of histone H2B whereas depletion of histone H2B transcripts did not inhibit an ongoing round of DNA synthesis. As the cell progresses from S phase, histone mRNA levels decrease (Marzluff and Pandey, 1988).

It is known that inhibitors of DNA synthesis decrease levels of histone transcripts (DeLisle *et al.*, 1983). UV-induced DNA damage inhibits DNA synthesis by blocking DNA fork displacement (Berger and Edenberg, 1986) and reduces the initiation of replication (Cleaver *et al.*, 1983; Painter, 1985). If UV (254 nm) exposure inhibits DNA synthesis in lens epithelial cells, then the

decrease of histone H3 and H4 transcripts after exposure to UV radiation observed in this study may be a result of inhibition of DNA synthesis. Studies by Griffiths and Ling (1989) showed that 5 h after exposure to 5 J/m² of 254-nm UV in SV40-transformed human cells, there was no longer a blockage of DNA synthesis. To confirm the correlation between DNA synthesis and expression of histone H3 after exposure of lens epithelial cells to UV radiation, further studies are needed.

UV radiation had an effect on the progression of lens epithelial cells through the cell cycle. The results suggest that UV radiation gives rise to a build-up of cells that have S phase DNA content, which coincides with findings of Lu and Lane (1993). When lens epithelial cells received 25 J/m² of UV, it appeared that there was also a build-up of cells in G1 phase arrest. In our study, inhibition of cell cycle progression similarly coincided with a decrease in RNA levels specific for histones H3 and H4. It is possible that the decrease in histone RNA may play a role in UV-induced cell cycle arrest.

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FIGURE LEGENDS

- Fig. 1 Relative expression of PKC-1, PKC-II, and PKC-III genes after exposure to 12.5 J/m^2 as measured by DNA-excess dot blots.
- Fig. 2 Relative expression of β -actin, γ -actin, α -tubulin, and vimentin genes following exposure to 12.5 J/m^2 of 254-nm radiation as measured by DNA-excess dot blots.
- Fig. 3 DNA hybridization analysis of histones H3 (A) and H4 (B), c-jun (C), PKC-I (D), and γ -actin (E) mRNA after exposure to 12.5 J/m^2 (lanes: C = unirradiated control; 1 = RNA collected 1 h following radiation exposure; 2 = RNA collected 3 h following radiation exposure; 3 = RNA collected 6 h following radiation exposure). Equal amounts of total RNA were separated on agarose-formaldehyde gels and transferred onto nitrocellulose. After hybridization with oligo-labeled DNA probes, blots were analyzed by PhosphorImager.
- Fig. 4 The graph depicts levels of RNA for histone H3 after exposures to different doses of UV radiation.

Fig. 5 Flow cytometric analysis of DNA content in lens epithelial cells after indicated exposures to UV radiation. Cells were harvested 1 h, 3 h, and 6 h after radiation exposure. The left-most peak in each histogram represents the G_1 population and the right-most peak represents the G_2 population. The top figure is the unirradiated control.

Table I. Cell Cycle Analysis: UV-induced Effects in N/N1003A*

Dose of 243-nm UV (J/m ²)	Time after exposure (h)	G ₁ (%)	G ₂ (%)	S (%)
0	control	58.2	22.2	19.6
5	1	57.1	19.2	23.7
	3	59.6	13.9	26.5
	6	54.9	7.6	37.5
12.5	1	58.0	19.1	22.9
	3	60.2	14.5	25.3
	6	64.1	3.9	32.0
25	1	57.3	19.1	22.8
	3	61.6	16.1	22.3
	6	69.3	3.9	26.8

*Determined by Partec analysis of fixed cells.

Fig 1

Changes in Lens Cell Gene Expression

Fig. 2

0 1 3 6

H3 Histone
UV
lens cells

Fig 3A

0 1 3 6

H4 Histone
UV
Lens cells

Fig 3B

0 1 3 6

c-jun
UV
lens cells

Fig 3C

0 1 3 6

PKC-I
UV
lens cells

Fig 3 D

0 1 3 6

γ -actin
UV
lens cells

Fig 3E

Fig 4

0

5 J/m² 12.5 J/m² 25 J/m²

Fig. 5