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Egr-1 inhibits apoptosis during the UV response: correlation of cell survival with Egr-1 phosphorylation

Ruo-Pan Huang[2], Yan Fan[2], Ian deBelle[1], Zhengyu Ni[1], Wynette Matheny[1] and Eileen D. Adamson[1,3]

[1] The Burnham Institute 10901, N. Torrey Pines Rd, La Jolla, California 92037, USA
[2] Molecular Medicine, Northwest Hospital, 120 Northgate Plaza, Suite 230, Seattle, Washington 98125, USA
[3] corresponding author: tel: (619)455-6480; fax: (619)646-3195; e-mail: eadamson@ljcrf.edu

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Abstract

UV irradiation of normal or immortalized cells induces a rapid increase in the expression of several transcription factors and is thought to serve a protective function. The human fibrosarcoma cell line, HT1080 clone H4, expresses almost undetectable levels of Egr-1 and does not respond to UV-C irradiation by the induction of Egr-1. The H4 cells are hypersensitive to UV which induces apoptosis and reduces clonogenicity. The introduction of exogenous Egr-1 into H4 (H4E9 and H4E4 cell-lines) confers protection from UV damage as measured by a number of assays. In both NIH3T3 (with inducible Egr-1) and H4E9 (constitutive Egr-1) cells, UV irradiation gave enhanced transactivation of Egr-1 reporters that correlated with *phosphorylated* Egr-1. Studies using inhibitors indicated that protein kinase-C and tyrosine kinases are involved in the anti-apoptotic effects of Egr-1 after UV damage. This is the first description of a biological effect of phosphorylated Egr-1.

Keywords: early growth response-1 transcription factor; UV-C response; clonogenicity; phosphorylation; HT1080 fibrosarcoma cells; Egr-1 response element; transactivation

Abbreviations: CAT, chloramphenicol acetyl transferase; Egr-1, early growth response gene-1; UV-C, ultraviolet of wavelength 200–290 nm

Introduction

Exposure to ultraviolet irradiation (UV) in mammalian cells triggers the UV response which is characterized by the induction of many genes (Fornace, 1992; Sachsenmaier *et al*, 1994a) including transcription factors such as Egr-1, AP-1 and NF-κB. Activation of these transcription factors by UV is mediated through oxidative stress and/or DNA damage and c-Ras is a component in the signal transduction pathway (Engelberg *et al*, 1994; Devary *et al*, 1991, 1993). We have shown earlier that stimulation of activated Ras expression leads also to increased Egr-1 expression (Huang *et al*, 1994b). In addition, the UV signal generates reactive oxygen intermediates near the membrane and these activate a number of growth factor receptor kinases such as the Epidermal Growth Factor Receptor (EGFR) (Huang *et al*, 1996) upstream of Ras. Others have shown that UV irradiation enhances AP-1 activity and induces the nuclear translocation of NF-κB (Radler-Pohl *et al*, 1993; Stein *et al*, 1989; Devary *et al*, 1992, 1993). Increased AP-1 activity has been shown to be implemented by the phosphorylation of the amino-terminal transactivation domain of c-Jun by the JNK ser/thr kinase (Hibi *et al*, 1993; Sluss *et al*, 1994; reviewed by Boulikas, 1995; Karin, 1994; Papavassiliou, 1994).

The irradiation of living organisms by ionizing or non-ionizing electromagnetic waves can inflict damaging changes in replication fidelity if genes become mutagenized. Therefore, most living beings have devised ways of limiting such damage. In bacteria, UV activates the SOS response which is characterized by a number of phenotypic changes including enhanced capacity for DNA repair as well as mutagenesis, inhibition of cell division and prophage induction (Holbrook and Fornace, 1991). In mammalian cells, the UV response has been demonstrated to suppress the immune response (Kripke, 1994) in addition to serving a protective function against UV damage (Devary *et al*, 1992; Engelberg *et al*, 1994; Holbrook and Fornace, 1991; Lu and Lane, 1993). However, precise mechanisms for any protective function are unknown, although cell cycle arrest (Wang and Ellem, 1994; Orren *et al*, 1995; DiLeonardo *et al*, 1994; El-Deiry *et al*, 1994), DNA repair (Petersen *et al*, 1995) and the apoptotic process (Caelles *et al*, 1994; Lowe *et al*, 1993) are receiving a great deal of attention. By the elimination of cells with damaged genes by apoptosis or programmed cell death, the population is normalized. This long-term protective effect is necessary to eliminate the mutagenized cell that by chance may have become growth deregulated. Tumor cells have escaped the normal rules of cell regulation in a variety of ways, and survive as a population when normal counterparts would not. In many cases p53 is known to be mutant as in H4 cells but the most striking characteristic of this human tumor cell line is the almost complete absence of Egr-1 expression. The data fit the hypothesis that the lack of Egr-1 is a major influence in the transformed character of this and other human tumor cell lines. We have shown previously that H4, a clone of HT1080 cells (Anderson *et al*, 1994), stably expressing exogenous Egr-1 become growth inhibited, less transformed *in vitro* and less tumorigenic in a dose dependent fashion (Huang *et al*, 1995). In this case, Egr-1 has a tumor suppressor activity and protects cells by slowing the cell cycle so that DNA repair can occur.

The present analysis concerns the damage or other responses that UV irradiation inflicts on cells *in vitro.* and

whether Egr-1 plays any role in these processes. The H4 line is ideal since it is incapable of eliciting Egr-1 expression after UV irradiation, unlike all other normal or immortalized cell lines tested. Using H4 cells and subclones that express high levels of exogenous Egr-1, we find that Egr-1 plays a different kind of role in cell protection after UV exposure. Egr-1 abrogates the apoptotic pathway so that more cells survive. To balance this effect, Egr-1 alters the cell cycle by becoming arrested in S and G2 phases. We present evidence that UV stimulates substantial phosphorylation of Egr-1 after irradiation of H4E9 cells as well as normal immortalized cells (NIH3T3). This modification considerably enhances Egr-1 transactivation activity in H4E9 and NIH3T3 cells. We hypothesized that phosphorylated Egr-1 is more effective than non-phosphorylated Egr-1 in the abrogation of apoptosis. In support of this, the use of inhibitors of protein kinase C (PKC) and tyrosine kinases that can inhibit the pathway leading to the phosphorylation of Egr-1, also reduce its transactivating activity as well as the anti-apoptotic role of Egr-1.

Results

Egr-1 enhances cell survival after UV irradiation

We demonstrated previously that reducing the endogenous Egr-1 levels in NIH3T3 cells using an antisense RNA expression vector, reduces cell survival in short term culture after UV irradiation (Huang and Adamson, 1995). To further extend this study, we turned the tumor cell line, HT1080 clone H4, which exhibits almost undetectable Egr-1 even after stimulation by serum, UV and TPA as shown in Figure 1 (lanes 1 to 5). Such a cell line represents an ideal system to analyze the function of Egr-1 by introducing an expression vector that expresses exogenous Egr-1 stably and to different levels in individual clones (Huang *et al*, 1995). H4N is a G418-selected control line transfected with the empty vector pFCS; H4E4 and H4E9 are two clones expressing high levels of exogenous Egr-1. Other clones expressing lower levels of Egr-1 had reduced ability to slow growth. The constitutive high level of expression of Egr-1 in H4E9 is shown in Figure 1 (lanes 6–10) and is not further inducible by serum or UV. The morphology and growth characteristics of the cells over-expressing Egr-1 are altered; the cells are larger and flatter, better growth regulated, less transformed (anchorage-independent and clonogenic assays) and are less tumorigenic (Huang *et al*, 1995). We asked whether constitutively expressed Egr-1 could also confer protection against UV-induced growth inhibition in this cell model.

To test short term effects, cell lines expressing no Egr-1 (H4 and H4N) and high levels of Egr-1 (H4E4 and H4E9) were treated with a (non-lethal) UV-C dose of 20 J/m^2. Following UV exposure, cell growth rates were determined by counting the number of cells up to 3 days later. As shown in Figure 2A, UV treatment significantly reduced the relative number of cells of parental (H4) and control cells H4N) compared with high Egr-1 expressing clones. The Egr-1-expressing cell lines survived doses of up to 20 J/m^2 with only 5–8% reduction in relative cell numbers compared to 34% reduction in the controls after 2 days. The difference was greatest at a UV dose of 20 J/m^2;

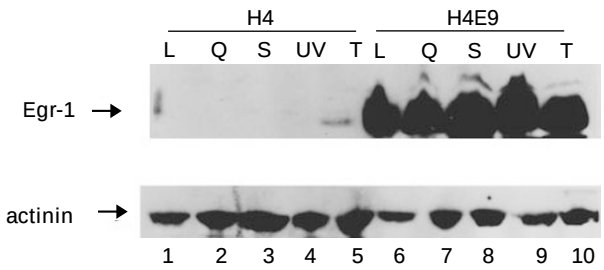


Figure 1 Immunoblot analysis of Egr-1 protein in human fibrosarcoma cell clones. H4 is a subclone that is highly tumorigenic and expresses little detectable Egr-1, lanes 1–5, even after stimulation with serum (S), ultraviolet-C irradiation (UV) or with tumor promoter TPA (T). L=log phase and Q=quiescent cells. H4 cells were transfected with an Egr-1 expression vector and a clone that constitutively expresses Egr-1 was selected, H4E9. Expression of Egr-1 in H4E9 cells is also uninducible. To indicate approximately equivalent loading of the lanes, the blot was reprobed for the cytoskeleton protein, α-actinin

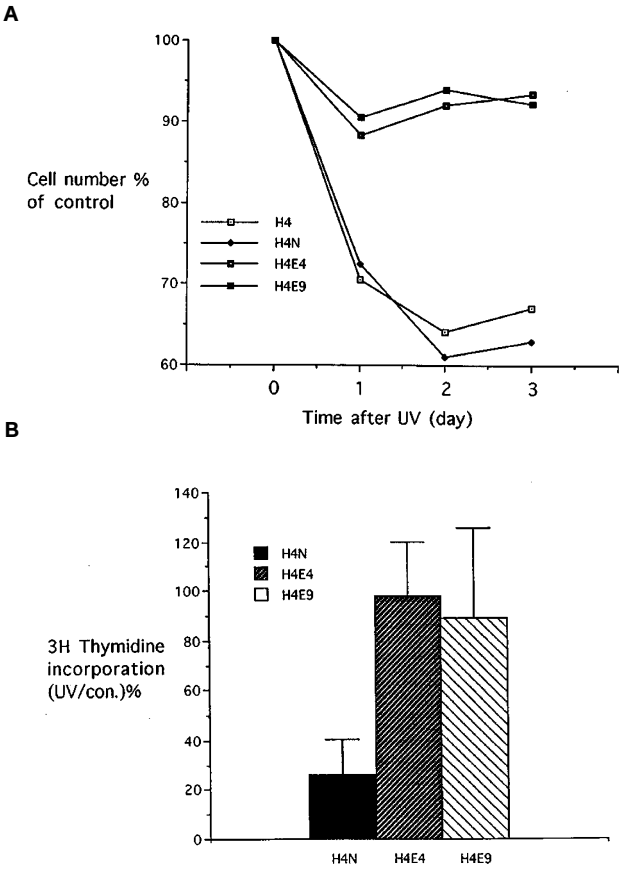


Figure 2 Cell growth after UV irradiation. (**A**) cell numbers were counted 1–3 days after 20 J/m^2 UV-C irradiation and expressed as a percentage of the non-irradiated cells. H4, parental cells; H4N G418-resistant control cells; H4E4 and H4E9 are two clones expressing high levels of exogenous Egr-1. (**B**) tritiated thymidine incorporation 2 days after irradiation (20 J/m^2) indicated that Egr-1-containing clones synthesized DNA at normal levels while H4N control cells were damaged and recovered only 20% activity

higher doses were lethal to all cell types (data not shown). The results were further confirmed by [^{3}H]thymidine incorporation assays (Figure 2B). Here, the protective effect of Egr-1 2 days after irradiation was more obvious with 70–75% higher rates of DNA synthesis in Egr-1-containing clones, H4E9 and H4E4 compared to the control line, H4N.

To test long term effects of Egr-1 after UV irradiation, we performed clonogenic assays in plastic dishes. A known number of cells were plated and then exposed to increasing doses of UV-C from 0 to 40 J/m^2. After 3 weeks in culture, the numbers of colonies formed were determined and compared with the number of colonies in unirradiated cells (Figure 3). The clones expressing Egr-1 had a greater fractional survival compared to parental and control clones. The results suggest that exogenous Egr-1 has a long-term protective function against UV irradiation and one mechanism could be on the rate of cell death.

Egr-1 protects cells from apoptosis after UV irradiation

The clonogenic assay suggested that Egr-1 protected H4E9 cells against apoptosis induced by UV irradiation, three different methods were applied to test this hypothesis. First, cells with or without UV irradiation were treated with Hoechst dye to stain DNA and nuclei in cells. Epifluorescent microscopic observation was used to detect cells with morphological changes including nucleoplasmic condensation, nuclear fragmentation and formation of apoptotic bodies.

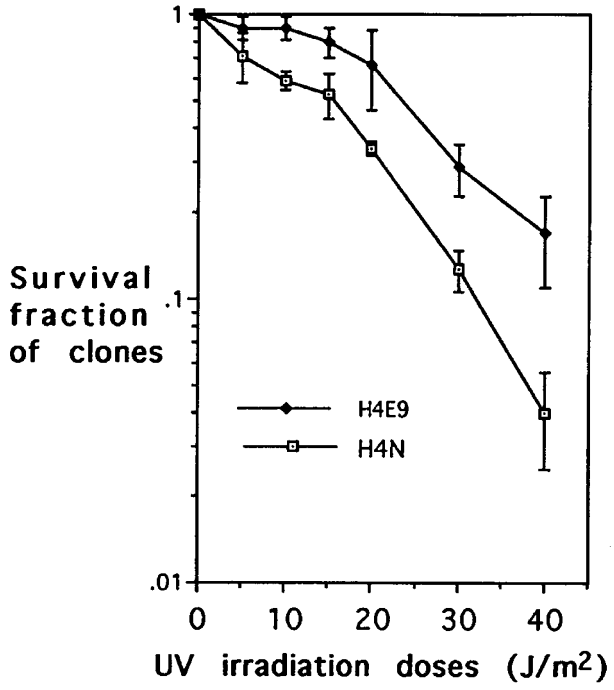


Figure 3 Survival fraction of clones following a range of doses of UV-C. Egr-1 was protective in the clonogenic growth subsequent to irradiation expressed as a fraction of non-irradiated cells 14 days after irradiation. Surviving clones were counted and are expressed on a logarithmic scale against UV dose

As shown in Figure 4A, high Egr-1-expressing clones display less apoptosis after UV irradiation (Figure 4A, right panel). The numbers of apoptotic bodies were counted and compared in Figure 4B. There were fewer apoptotic cells in Egr-1-expressing cells (7%) compared to control cells (17%, $P<0.01$).

The second type of assay is more sensitive to DNA damage and adaptable to quantification. Cells were labeled with [^{3}H]thymidine extensively before exposure to UV (the same 20 J/m^2 dose was used except where indicated). The radioactivity released from the cells was determined 24 h after exposure to UV. The results were normalized to untreated cells (Figure 5A). Again the same relationship between high levels of Egr-1 and lower apoptosis (59% of control cells) was observed. In the third assay, genomic DNA was extracted from UV treated and untreated cells and separated on 2% agarose gels. As shown in Figure 5B (right panel), UV-treated H4N cells exhibited increased levels of fragmented and low mol. wt. DNA, in a dose-dependent manner, visible as a DNA ladder and as a smear. Low mol. wt. DNA was detectable in all UV-treated cells but no laddering occurred in H4E9 cells (Figure 5B, left panel) whereas it was readily visible in H4 cells under the same conditions. Taken together, these data suggest that the process of programmed cell death is activated upon exposure of cells to UV irradiation and that Egr-1 inhibits the apoptotic pathway.

Egr-1 functions by increasing the fraction of cells in S and G2 phases of the cell cycle after UV

Cells were analyzed by fluorescence activated cell sorting (FACS) for the fraction of cells that remained in the G1 or G2 phases of the cell cycle that would account for the slower

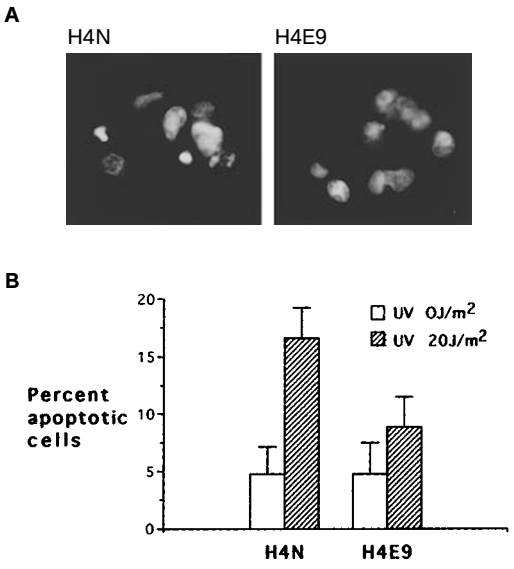


Figure 4 Apoptosis observed by Hoechst staining (**A**) of nuclei following irradiation. (**B**) the abnormal nuclei were counted and are expressed as % of cells counted in cultures of control H4N and H4E9 cells

growth rates of the Egr-1-expressing cells. In unirradiated cells there was little difference between the two cell lines in the percentage of cells in each phase of the cell cycle (Figure 6, table), although the cell cycle progressed more slowly in H4E9 cells. In these analyses, 24 h after a UV dose of 40 J/m^2, an apoptotic peak that denotes fragmented DNA, was prominent in H4 cells (30%) and the G1 phase was depleted as cells underwent apoptosis. Irradiated H4E9 cells showed a more persistent S+G2/M phases compared to H4N and H4 cells and less apoptosis, in 3 experiments (a representative experiment is shown). The results suggest that the presence of Egr-1 protein expression in H4E9 cells leads to increased entry into S and G2 pausing, perhaps preventing apoptosis in this manner. The increased proportion of cells in S-phase is continued for at least 2 days as indicated by the data in Figure 2B.

Egr-1 is phosphorylated in response to UV irradiation

Our previous data showed that UV-C rapidly induces Egr-1 expression in NIH3T3 cells (Huang and Adamson, 1995). The increase in accumulated Egr-1 protein starts less than 10 min after irradiation and is maximal at 1–2 h before declining to base-line levels. The induction is at least partially transcriptional (data not shown) and, in addition, the protein is noticeably modified since it migrates at slower rates. In contrast, although serum and tumor promoters also induce Egr-1, very little of the slower migrating forms are seen. These slower migrating species are probably related to post-translational modifications such as phosphorylation, glycosylation or oxidation. To test whether phosphorylation of Egr-1 occurs, we examined

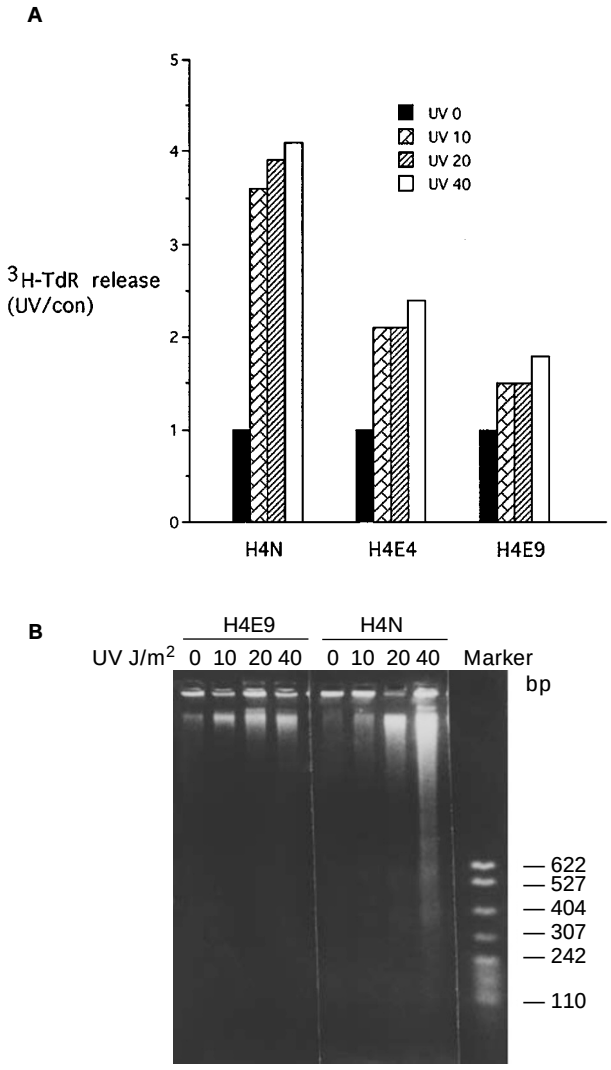
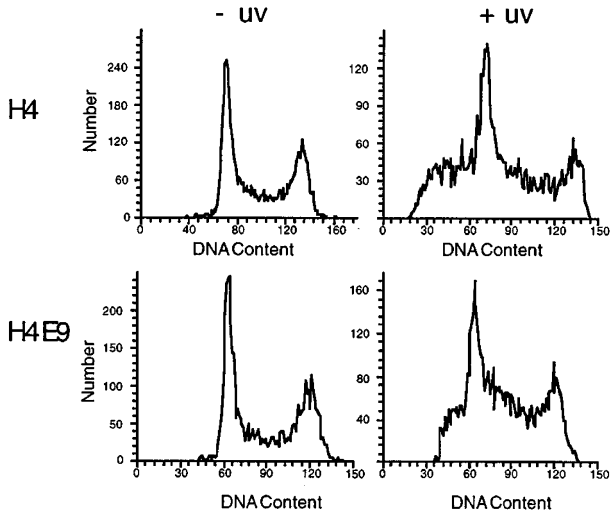


Figure 5 Apoptosis assays. (**A**) release of tritiated thymidine after UV irradiation. Cell DNA was extensively labeled by metabolic incorporation of tritiated thymidine before exposure to a range of doses of UV. DNA damage was dose-dependent, but was less in cells containing high levels of Egr-1, H4E4 and H4E9. (**B**) low Mol. Wt. DNA was extracted from cells after UV irradiation and analyzed on 2% agarose gels to show the pattern of breakdown products stained with ethidium bromide. H4N cells display detectable low Mol. Wt. DNA after UV irradiation while H4E9 cells were protected by the presence of Egr-1



	H4		H4E9	
uv	-	+	-	+
G1	41	25	40	26
S	29	28	30	38
G2/M	30	16	30	23
Apop.	0	30	0	13

Figure 6 Cell Cycle Analysis. Cell cycles were analyzed in log phase growth (left panels) and 24 h following exposure of H4 (upper panels) and H4E9 (lower panels) cells of 40 J/m^2 UV-C (right panels). One of three similar cell cycle analyses is shown. The left-most shoulder in the irradiated samples represents apoptotic cells with subdiploid DNA content. The highest peak contains cells with a GO-G1 DNA content; whereas the right-most peak represents cells with G2/M DNA content. The region between the two peaks represents cells progressing through S-phase. The table shows the percentage of cells in each phase of the cell cycle

the effects of UV on NIH3T3 cells incubated in the presence of [^{32}P]orthophosphate for 2 h immediately after exposure to UV-C. As shown in Figure 7A, there was a large increase in immunoprecipitable phosphorylated Egr-1 that migrated at retarded electrophoretic mobility. This decrease in mobility was also observed in immunoprecipitates when cells were incubated with [^{35}S]methionine (data not shown) or when immunoblotting was performed (Huang and Adamson, 1995). Slow-migrating forms of Egr-1 can be detected as long as 12 h after UV irradiation (data not shown). Our previous measurements of the induction of Egr-1 in NIH3T3 cells by serum or by TPA gave 7–tenfold increase in Egr-1 protein while the levels of slow-migrating phosphorylated Egr-1 were not increased by serum and only slightly increased by TPA (Huang *et al*, 1994b; Huang and Adamson, 1995). The results here suggest that UV significantly enhances both the Egr-1 protein level and its phosphorylation in NIH3T3 cells.

Since exogenous Egr-1 expression in H4E9 cells is invariant (Figure 1), we wished to determine if this preformed Egr-1 is also modified by UV irradiation of H4E9 cells. Figure 7B (upper left and right panels) shows

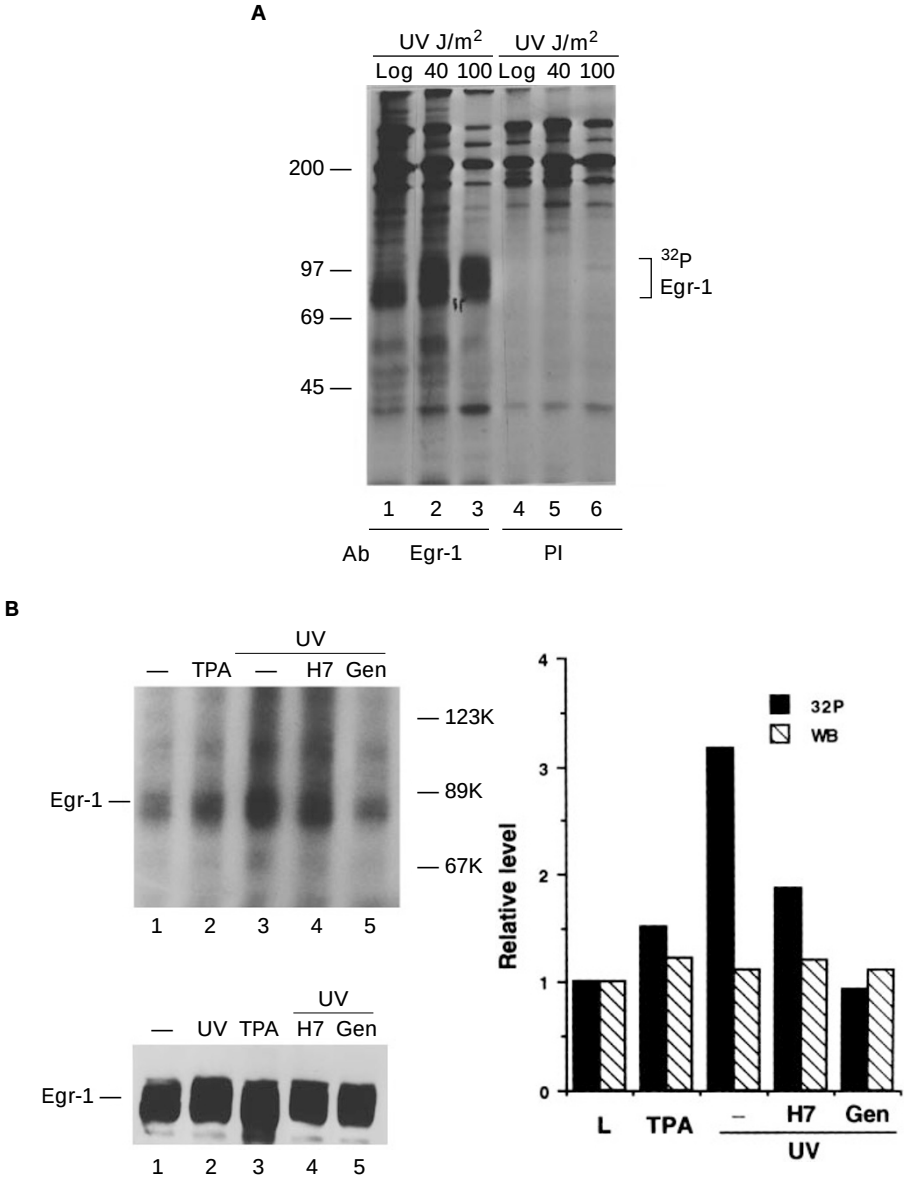


Figure 7 Immunoprecipitation analysis of phosphorylated Egr-1. (**A**) NIH3T3 cells were metabolically labeled with [^{32}Pi] immediately following exposure to two different doses of UV irradiation. After 2 h, the cells were harvested and lysates were analyzed for the presence of radiolabeled Egr-1 using either the preimmune serum (lanes 4–6), or anti-Egr-1, (lanes 1–3). Cells in logarithmic growth produced phosphorylated Egr-1 and this was induced 4- to 5-fold after UV irradiation. (**B**) H4E9 cells were pretreated with H7 (30 μM) and Genistein (100 μM) for 30 min in lanes 4 and 5 and metabolically labeled with [^{32}Pi] immediately following exposure to UV or addition of TPA (25 μM) for 2 h, followed by immunoprecipitation with antibodies to Egr-1. Quantification (right) by radioactivity counting showed that UV enhanced phosphorylation more than three times compared to unstimulated cells. The phosphorylation was partially inhibited by PKC inhibitor, H7, and tyrosine kinase inhibitor, genistein (G). Immunoblotting (bottom left) showed that steady state levels of Egr-1 did not change during these treatments

that the small amount of phosphorylated Egr-1 in log phase cells was increased 1.5-fold by the addition of TPA to the medium for 1 h and >threefold by UV irradiation. The level of phosphorylation was somewhat reduced by co-incubation with H7 (a PKC inhibitor) and almost completely inhibited by the addition of genistein (a tyrosine kinase inhibitor). Hence, PKC and tyrosine kinases appear to be involved in the phosphorylation of constitutively expressed Egr-1. Genistein could be inhibiting the RTKs such as EGFR that are involved with signal generation after UV (Huang *et al*, 1996). However, in H4E9 cells, the total Egr-1 protein level remained unchanged as observed in Western blotting (Figure 7B, bottom left), therefore the results together suggested that UV irradiation of cells leads to the extensive phosphorylation of pre-existing Egr-1.

Phosphorylated Egr-1 activates transcription more efficiently than non-phosphorylated

We have previously documented that the phosphorylated forms of Egr-1 bind to the DNA target sequence to a greater degree compared to under-phosphorylated forms (Huang and Adamson, 1994). Irradiation induced the hyper-phosphorylated forms of Egr-1 and indicated that phosphorylated forms may be more active. To examine the transactivating activity of phosphorylated Egr-1, we used NIH3T3 cells that had been stably transfected with a reporter plasmid containing two Egr-1 binding sites, 2xEgr-1BSCAT, or with empty vector CAT-2 (as a control) together with pSVneo. Cells resistant to G418 were isolated and compared for their ability to activate chloramphenicol acetyl transferase (CAT) activity under various conditions. If active Egr-1 is produced after irradiation with UV, serum or TPA, it should stimulate CAT activity through binding to the Egr-1 binding sites. As shown in Figure 8, UV significantly induced 2xEgr-1BSCAT2 activity (11–18-fold) compared with serum (S) (twofold) or TPA addition (up to 1.7-fold) and this effect was dose and time-dependent (Figure 8A and C). UV irradiation (40 J/m^2) also induced empty vector CAT activity (Figure 8B) to a small extent (2.5-fold). The results were further confirmed by transient transfection CAT assays (data not shown). The increased CAT activity after UV was not due to the induction of higher total Egr-1 protein levels by UV because Egr-1 levels were equally inducible by serum or TPA, but these agents did not greatly increase the transactivating activity of Egr-1. Rather, the increased transactivating activity of Egr-1 after UV irradiation was correlated with the level of slower-migrating *phosphorylated* Egr-1.

To distinguish between Egr-1 induction and Egr-1 phosphorylation by post-translational mechanisms, we took advantage of H4E9 cells in which the constitutively high exogenous Egr-1 level is not further inducible by UV, serum or TPA. The plasmid EBS1^{3}fosCAT vector containing three Egr-1 binding sites, or a control vector pΔ56fosCAT, was transfected into H4E9 cells. After 16 h, cells were treated with UV, serum or TPA and CAT activities were determined 24 h later. Figure 9A shows that UV remarkably enhanced the already high basal CAT activity of the reporter construct by increasing transactivating activity of the Egr-1 binding site 6.5-fold (control vector was not stimulated). Furthermore, preincubation of H4E9 cells with tyrphostin, lavendustin (tyrosine kinase inhibitors) or H7 (PKC inhibitor), inhibited the transactivating of Egr-1 by 40–60% (Figure 9C, 4 left lanes) down to the basal activity of the empty CAT vector (Figure 9C, 4 right lanes). UV did not induce EBS1^{3}fosCAT activity in control H4N cells (Figure 9B) where Egr-1 levels were extremely low or absent. Increased Egr-1 protein stability *per se* did not contribute to the enhanced CAT activity since the protein stability was almost the same for total Egr-1 produced after UV and serum stimulation (data not shown). We concluded that hyperphosphorylated Egr-1, such as that produced after UV irradiation, is a more powerful transactivator of its target genes compared to under-phosphorylated Egr-1.

The function of Egr-1 is modulated by both PKC and tyrosine kinases

We have shown earlier that both protein kinase-C (PKC) inhibitors as well as tyrosine kinase inhibitors attenuate UV-

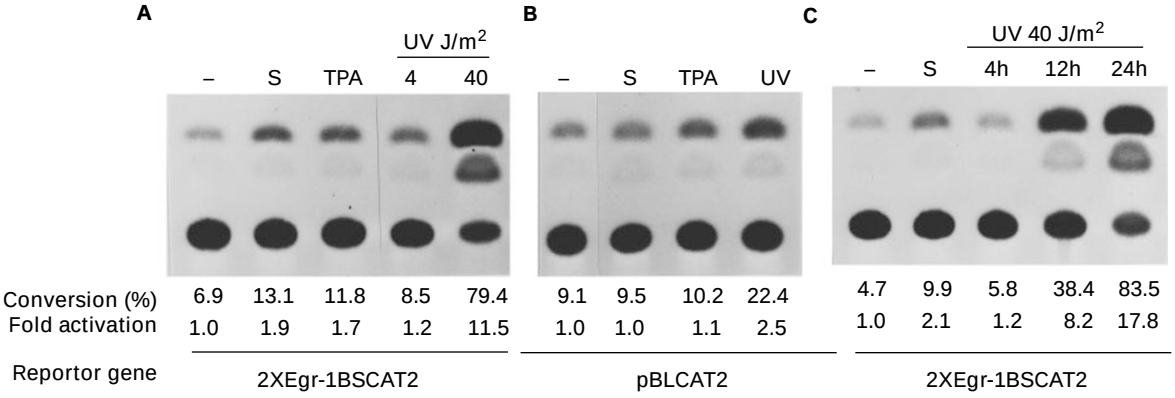


Figure 8 Phosphorylated Egr-1 activates its binding site with increased efficiency compared to non-phosphorylated Egr-1. NIH3T3 cells were transfected with reporter genes and selected for constitutive activity with G418. The 2xEgr-1 binding site, GCGGGGGCG, was ligated to the reporter BLCAT-2 construct to test for the transactivating activity of Egr-1 measured 24 h later (**A**) the addition of various stimuli to the cells: S, 20% serum; 0.2 μM TPA; UV irradiation at 4 and 40 J/m^2. In (**B**) the cells expressed the empty vector, and 40 J/m^2 was used. In (**C**) the time course of activation was tested by harvesting cells at the times indicated for up to 24 h. The values were expressed as fold-induction compared to the non-stimulated cells in log phase

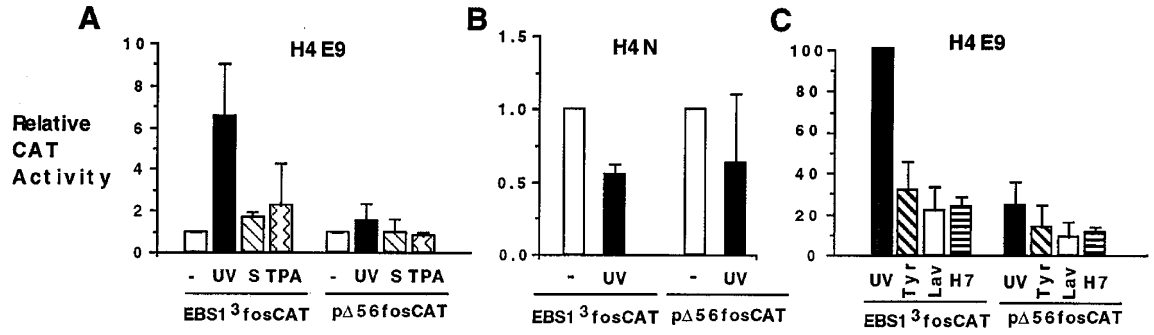


Figure 9 UV-induced enhanced Egr-1 transactivating activity in H4E9 cells. pEBS1^{3}fosCAT reporter gene containing three Egr-1 binding sites, and control vector, pΔ56fosCAT, were transiently transfected into H4E9 or H4N control cells. The CAT activities were determined as indicated in the Methods section and quantified using the phosphorimager system. The experiment was repeated twice with the same results. (**A**) relative CAT activity after treatment of cells with UVC (40 J/m^2), serum (20%) or TPA (0.2 μM) (**B**) H4N cells were UV irradiated after transfection. (**C**) H4E9 cells were pretreated with kinase inhibitors 30 min before UV irradiation. H7 (30 μM, a PKC inhibitor), tyrphostin (10 μM, a tyrosine kinase inhibitor) and lavendustin A (1 μM, a tyrosine kinase inhibitor)

induced phosphorylation of Egr-1 protein (Figure 7B, and Huang and Adamson, 1995). To test the role of phosphorylated Egr-1 in the protection of cells against UV damage, we used inhibitors of PKC and tyrosine kinases in growth assays in NIH3T3 cells. In these studies, 48 h pre-exposure to TPA was used to down-regulate PKC. Given a UV dose of 20 J/m^2, followed by a brief (30 min) exposure of the cells to the PKC inhibitor, H7, or to the tyrosine kinase inhibitors, genistein and tyrphostin, the ability of the cells to grow was subsequently tested in two ways. Figure 10A shows that all of these inhibitors significantly decreased the growth rate as measured by cell mass 2 days after UV irradiation. The long-term effects of these inhibitors on clonogenic growth of cells after irradiation (Figure 10B) was also measured. Kinase inhibitors reduced clonogenicity to 29–47% of irradiated cells that were not treated with inhibitors. The combination of protein kinase C inhibitor, H7, plus either tyrphostin or genistein was additive, reducing clonogenicity to 8% that of untreated, irradiated cells, suggesting that two Egr-1 phosphorylating signal pathways are operating independently during the cell's response to UV injury.

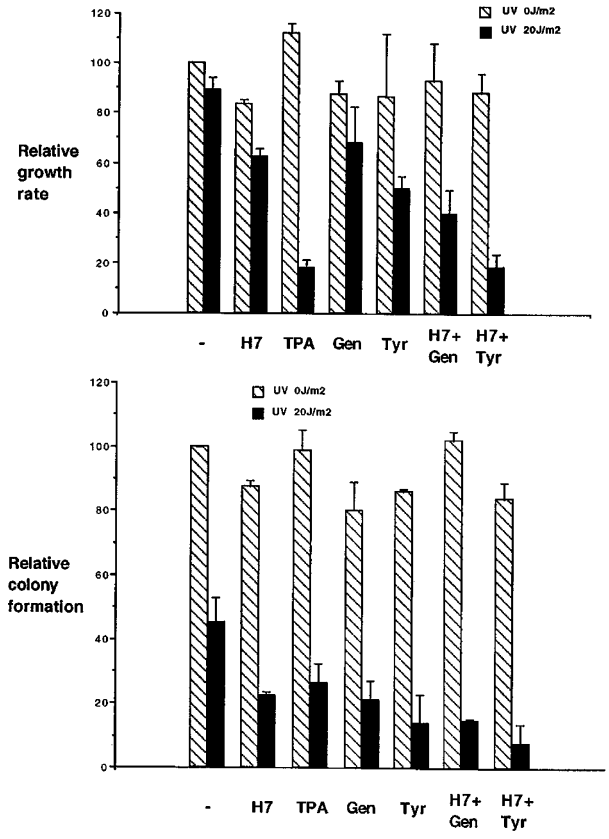


Figure 10 The effect of kinase inhibitors on cell growth rates after UV irradiation. Growth was measured 2 days after irradiation and compared with non-irradiated cells. Inhibitors were added 30 min before UV exposure (except for TPA which was added for 48 h earlier to down-regulate PKC activity) and growth was then measured. (**A**) cell mass after 2 days in 96-well plates (MTT assay, see the Methods Section). (**B**) clonogenic growth 2–3 weeks after seeding cells in plastic tissue culture dishes

Discussion

A summary of the strategy used in these studies is presented in Figure 11. The data presented here suggest that UV irradiation induces substantial hyperphosphorylation of Egr-1 which subsequently enhances Egr-1 transactivation ability. It has been reported previously that Egr-1 is a nuclear phosphoprotein (Grover-Bardwick *et al*, 1994; Cao *et al*, 1990), however, the biological significance of this modification was unknown. Recently, we observed that the phosphorylated forms of Egr-1 bound to DNA more efficiently than non-phosphorylated (Huang and Adamson, 1994). We also found that Egr-1 as well as JunB, JunD and c-Jun were strongly phosphorylated in NIH3T3 cells conditionally over-expressing v-*sis* oncogene (Huang *et al*, 1994a). Results from other groups showed that the application of the phosphatase inhibitor, okadaic acid, to mouse fibroblasts cells increased the transactivation of the Egr-1 gene and increased the phosphorylated forms of the protein (Cao *et al*, 1992). All of these observations support the notion that phosphorylated modifications of Egr-1 (only Ser and Thr appear to be phosphorylated) may have profound importance to its biological functions. Our results suggest that these functions may be realized *via* increased transactivation ability. One of

the target genes of Egr-1 in its growth inhibitory role is the TGFβ gene (Liu *et al*, 1996). Although we have not yet identified the relevant target genes for anti-apoptosis, several UV responsive genes (c-Ha-*ras*, c-*abl*, *jun*-B, c-*fos*, mouse metallothionein I, TNFα) contain Egr-1 binding sites (Holbrook and Fornace, 1991) and phosphorylated Egr-1 may subsequently activate or inhibit such target genes (Krämer *et al*, 1994). The UV-induced phosphorylation of Egr-1 involves both the PKC and tyrosine kinase pathways because the inhibition of these kinase activities significantly reduced Egr-1 phosphorylation (Figure 7B), transactivating ability (Figures 8 and 9) and cell survival after UV (Figure 10).

In mammalian cells, UV exposure turns on the UV response process which is characterized by increased levels of transcription factors including Egr-1 and AP-1 and increased stabilization of p53 (Liu *et al*, 1994). The biological function of the mammalian UV response is just beginning to be understood. By using tyrosine kinase inhibitors, Devary *et al* (1991) showed that UV-elicited AP-1 response has protective functions. Here, we provide evidence, at the individual gene level, that Egr-1 functions in the protection of cells against UV damage that leads to decreased clonogenicity. There are at least two components that could contribute to the protective effect of Egr-1. One is that constitutive Egr-1 expression slows the cell cycle (especially in the G2 phase, data not shown) so that DNA repair enzymes have a greater ability to replace damaged DNA. In addition, after UV irradiation, the ratio of cells in the S and G2 phases of the cell cycle increase significantly in H4E9 cells but not in H4N control cells (Figure 4, Figure 6 Table). The second is that the stimulus to enter the apoptotic pathway in radiosensitive parental H4 cells, is avoided in Egr-1-expressing H4 cells. As a result, the higher fraction of cells survive in Egr-1-containing cells compared to Egr-1-minus

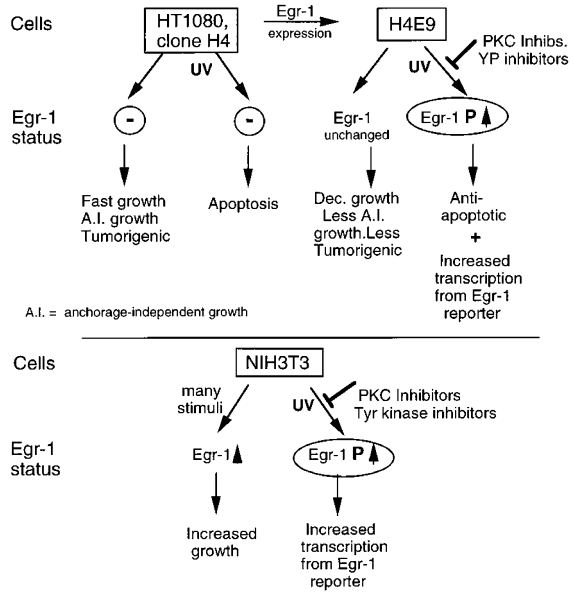


Figure 11 Summary of cell lines and strategy used to show that phosphorylated Egr-1 has elevated transactivating activity that correlates with anti-apoptotic activity

parental H4 cells. Our conclusion is also supported from other studies in which pretreatment of the human astrocytoma cell line, G18, with taxol enhanced radiosensitivity. Careful examination of seven immediate early genes including Egr-1, c-*jun* and c-*fos*, revealed that only Egr-1 was significantly inducible by radiation in G18 cells and the induction was blocked by pretreatment of cells with taxol (Gubits *et al*, 1993). Interestingly, the UV response pathway involving Ras and AP-1 (and perhaps including Egr-1) is highly conserved between yeast and mammals (Engelberg *et al*, 1994), although the evolutionary significance of the UV-induced process is unclear. Perhaps the UV response in eukaryotic cells represents an 'SOS' type of response to UV, designed to restore the balance between UV damage and the normality of the cell population.

Previously, we have shown that both PKC and tyrosine kinase activities are necessary intermediates in cell survival after UV irradiation. The results here are consistent with the report of Devary *et al* (1992) that tyrosine kinase inhibitors greatly enhance the cytotoxic effect of UV-C in Hela cells. Evidence from Hallahan *et al* (1992) which showed that inhibition of protein kinase C potentiates cell killing by ionizing radiation also complements our observation. The role of tyrosine kinase in the UV signal transduction pathway has been well documented. Several tyrosine kinases, such as EGF-receptor (Sachsenmaier *et al*, 1994b; Huang *et al*, 1996) and v-*src*, (Devary *et al*, 1992) have been identified in the UV signal cascade. The role of PKC is much more complicated. By employing broad specificity protein kinase inhibitors such as H7, other groups (Buscher *et al*, 1988; Hallahan *et al*, 1991; Papathanasiou *et al*, 1991) also observed that PKC is involved in the UV response. PKC has been demonstrated to activate the gene products of c-*raf* and c-*mos* proto-oncogenes which are serine/threonine protein kinases. Particularly c-*mos* has been shown to participate in PKC-mediated signal transduction pathways. However further experiments are needed to define the specific role of PKC in UV signaling.

Finally, the more challenging question we are facing comes from our recent finding that Egr-1 negatively regulates human tumor cell growth. Currently, we hypothesize that Egr-1 performs distinct functions dependent on its phosphorylation states and also serves several roles in cell cycle regulation. For example, cells carrying Egr-1 appear to arrest the cell cycle in S and G2 after UV irradiation, thereby allowing cells to repair any DNA damage. As a result, cells might be expected to increase in survival after UV irradiation. On the other hand, cells lacking Egr-1 will proceed to programmed cell death. Consequently, these cells might be expected to have increased sensitivity to UV irradiation. This idea is supported by our finding that Egr-1 can increase cell survival by inhibiting the apoptotic pathway, by arresting cell cycling and by allowing cell clonogenicity to proceed at higher levels than in the absence of Egr-1. The data support a model that phosphorylated Egr-1 is the form that is most able to protect cells after damage caused by UV irradiation.

Materials and Methods

Cell culture and materials

NIH3T3 cells were grown in DME containing 5% CS at 37°C and 5% CO_2 HT1080 derived clones H4 (Frisch and Francis, 1994), H4N, H4E4 and H4E9 (Huang *et al*, 1995) were maintained in DME supplemented with 10% FCS at 37°C in 5% CO_2. UV irradiation was performed as described elsewhere (Huang and Adamson, 1995). H7 and TPA were obtained from Sigma Chem. Co. (St. Louis, MO). Tyrphostin, lavendustin and genistein were purchased from Gibco-BRL (Gaithersburg, MD). Stock solutions were prepared as below: genistein, 20 mM in DMSO; lavendustin, 10 mM in water; H7, 30 mM in water; TPA, 0.25 mM in DMSO and tyrphostin 10 mM in DMSO. The working concentrations were 20 μM, 1–2 μM, 10 μM, 0.2 μM and 10 μM, respectively.

Plasmid construction

Plasmid 2xEgr-1BSCAT-2, containing two copies of the Egr-1 DNA binding site, was created by cloning of synthetic oligonucleotides containing 5′-GATCTCCTCGCCCCCGCGAGT into the *Bam*HI site in pBLCAT-2. The correct orientation, copy number and identity of clones were confirmed by dideoxynucleotide sequencing. The reporter plasmid, pΔ56fosCAT, containing the murine minimal fos reporter upstream of the chloramphenicol acetyl transferase gene, and EBS1^{3}fosCAT with three copies of a high affinity Egr-1 binding site, have been described (Gashler *et al*, 1993).

Transfection

NIH3T3 cells (1×10^6) were seeded into 100 mm tissue culture dishes 20 h prior to transfection. 20 μg of CAT vectors (pBLCAT-2 or 2xEgr-1BSCAT) plus 2 μg of pSVneo (for selection with G418) were co-transfected by calcium phosphate precipitation. After incubation overnight, the medium was replaced with fresh medium. Forty hours after transfection the cultures were trypsinized and seeded at a 1 : 4 ratio for selection with G418 at 400 μg/ml. Three weeks later, all the clones so formed were pooled together for further experiments. For transient expression studies, cells were harvested 40–48 h after transfection.

CAT assay

The stably transfected cells were seeded at 2×10^5 cells on a 60 mm plate. Twenty four hours later, cells were treated with UV (20 J/m^2) or TPA (0.2 μM). For serum stimulation, cells were incubated in DME containing 0.5% CS for 24 h and then 20% serum was added. For all types of stimulation, cells were harvested 24 h after transfection, lysed and assayed for CAT activity as described earlier (Huang *et al*, 1994b).

Immunoprecipitation

Cells were metabolically labeled by incubation in phosphate-free DME containing Tris-buffered dialyzed serum and 0.5 μCi of [^{32}P]inorganic phosphate (ICN, Irvine, CA) immediately after UV irradiation or addition of TPA. Equal amounts of radioactivity were subjected to immunoprecipitation using a rabbit antibody to an Egr-1 fusion protein product as described earlier (Huang and Adamson, 1993).

Western blotting

Egr-1 protein in cell lysates was assayed by immunoblotting as described (Huang *et al*, 1994b). Equal loading of the lanes was verified by reprobing the washed membrane with an antibody to α-actinin (Sigma Corpn., St Louis, MO).

Growth assays following irradiation

To test clonogenicity on plastic substrates, colony-forming assays were done by seeding 500 (NIH3T3 and H4N) or 1000 (H4E9) cells per 60 mm plate. The plates were irradiated with the indicated dose of UV-C provided by a Stratolinker 420, and culture continued with feeding every 3 days. After 2 to 3 weeks, the plates were washed in PBS, fixed in 4% formaldehyde-PBS and stained with Giemsa solution. Colonies containing >50 cells were counted and analyzed statistically by the students two-tailed *t* test.

Growth rates by cell counting assays. Five $\times 10^4$ cells H4, H4N, H4E4 and H4E9 were seeded in each well of a 6-well plate. After 20–24 h, cells were treated with indicated amounts of UV-C and allowed to grow for varying times. The trypsinized cells were counted using a Coulter counter. Assays were performed in triplicate and repeated at least twice.

Non-radioactive cell proliferation assay (MTT, Promega, Madison, WI). The tetrazolium dye end product of MTT assays was measured in a 96-well plate. NIH3T3 cells were seeded in 6-well plates at 5×10^4 per well and cultured overnight. Cells were irradiated with 20 J/m^2 after pretreatment with different inhibitors for 30 min (except that TPA treatment was continued for 48 h. Cells were trypsinized, suspended in 1 ml of medium containing inhibitors and transferred to 96 well plates (100 μl/well). After 2 days, cells were stained and processed as described by the manufacturer. The values of absorbances at 600 nm were plotted as a measure of the relative number of cells. Each assay was done in quadruplicate and repeated at least twice.

Tritiated thymidine incorporation. Five $\times 10^4$ cells were seeded in 6-well plates for culture overnight and then subjected to 20 J/m^2 UV-C irradiation. Two days later, the cells were treated with 1.0 μCi/well tritiated thymidine for 2 h. The cells were washed, fixed in Carnoy's fixative (methanol:glacial acetic acid, 3 : 1), dissolved in 0.3 M NaOH and counted in a scintillation counter. To assay for apoptosis, cells were extensively labeled by culture with tritiated thymidine overnight, the cells were washed with PBS three times and then exposed to UV irradiation at the indicated dose. Culture was continued for 24 h. The amount of tritium released into the supernatant was determined by counting aliquots in a scintillation counter.

Cell cycle analyses

Cells were harvested in logarithmic growth phase and seeded in 100 mm tissue culture plates at 5×10^5 to 10^6/dish. The next day, the medium was removed and cells were exposed to UV-C irradiation at various doses, the same medium was replaced and culture was continued for various times before harvesting. Cells were washed twice in PBS and detached in Ca/Mg-free PBS containing 1 mM EDTA. The cells were triturated to disaggregate, centrifuged, resuspended and fixed in 70% ethanol. The DNA in cells was stained by exposure to propidium iodide and cells were analyzed in a fluorescence activated cell sorter (Model FACSort, Becton Dickinson).

Apoptosis assays

Cells were fixed in Carnoy's solution (methanol:glacial acid, 3 : 1) and stained with 5 μg/ml of bisbenzimide trihydrochloride (Hoechst 33258) for 20 min. A Nikon Biophot microscope was used to observe nuclei

and fragmented nuclei. Counts were made of at least 500 nuclei in each cell line. The experiment was repeated once and statistical analyses made.

DNA fragmentation: 1×10^6 of H4N and H4E9 cells were seeded on 100 mm plates and incubated overnight. Cells were exposed to UV-C as indicated. After 24 h, cells (detached and attached) were harvested. Cells were lysed in 0.5% Triton X-100, 10 mM EDTA and 10 mM Tris pH 7.4 and low molecular weight genomic DNA was extracted by phenol-chloroform three times as described (Frisch and Francis, 1994).

Tritiated thymidine release (see above in Growth assays).

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