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Inhibition of Radiation-Induced Apoptosis *In Vitro* By Tumor Promoters

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The manner in which cells die following toxic stress or trauma has been divided into two general mechanisms: first, *necrotic* death associated with structural and biochemical degeneration of cell integrity; and second, *apoptosis* or programmed death which is associated with early endonucleolytic fragmentation of duplex DNA presumably a consequence of specific gene expression (see 1, 2). The physiological process of programmed cell suicide is directly associated with the regulation of proliferation, differentiation, cell aging *in vitro*, and cell-mediated immunity (3-5).

Williams, Little and Shipley (6) reported that widely differing modes of cellular trauma could initiate a specific pattern of DNA degradation preceding cell death. Initiation of apoptosis is dependent on both RNA and protein synthesis (7) supporting the concept that cytotoxic stress initiates a process of gene expression leading to the synthesis of protein(s) responsible for subsequent genomic degradation. These stress modalities include chemical toxicity, physiological stress (i.e. heat, osmotic stress) and radiation (8-11).

Our laboratory has proposed that tumor promoters such as the phorbol ester, TPA, and the indole alkaloid, DHTB, specifically inhibited the normal physiological process of apoptosis in normal cells *in vitro* (12,13). We wish to report data that indicates that tumor promoters inhibit programmed cell suicide in cultures of C3H-10T1/2 cells following either acute serum removal,

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Abbreviations: TPA, 12-O-tetradecanoylphorbol-13-acetate; DHTB, dihydroteleocidin B; ^{3}H-dThd, ^{3}H-thymidine; Gy; Gray.

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exposure to ionizing radiation, or incorporation of high specific activity ^{3}H-dThd into DNA.

We previously reported that TPA, but not the 4(O)-methyl analog, had an apparent influence on cell survival and could modulate G_1 substate transitions and S phase entry kinetics (17-21). It is of interest to extend these studies to other types of stress-induced breakdown and to confirm that the DNA fragmentation and subsequent death are related to programmed cell suicide. The two preliminary criteria used were DNA fragmentation patterns consistent with specific-site directed endonucleolytic strand breakage (12), and sensitivity to inhibition by low concentrations of cycloheximide. In addition to initiation of DNA fragmentation by serum withdrawal, radiation exposure was used in order to better define the cytotoxic stress. Radiation exposure was introduced either in the form of "soft beta" irradiation by incorporation of high specific activity ^{3}H-dThd into DNA in exponentially proliferating cultures, or direct exposure to gamma radiation using a ^{137}Cs irradiator.

MATERIALS AND METHODS

Cells: Cultures of C3H-10T1/2 (CL8) mouse fibroblasts were maintained as described (14) using Basal Eagle's Medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (Sterile Systems, Logan, UT). Growth medium was renewed every 3-4 days. Confluence was reached at 8-10 days, and postconfluent cells were used for the experiments (48-72 hours after the time of confluence). Experiments were performed on duplicate cultures in 60 mm diameter culture plates (Falcon Plastics).
Drugs: DHTB was a gift of T. Sugimura and H. Fujita (National Cancer Research Institute, Tokyo). TPA and analogs were obtained from Chemical Carcinogens, Eden Prairie, MN. 3H-Thymidine (3H-dThd) was purchased from ICN. Cycloheximide (reagent grade) was obtained from Sigma Chemicals.

DNA fragmentation is measured as previously described by Kanter and Schwartz (15,16) and a fragmentation index is calculated as the quotient of the radioactivity extracted from the single stranded DNA and the total amount extracted from both single and double stranded DNA.

RESULTS

Serum deprivation: Replicate cultures of exponentially proliferating 10T1/2 cells were transferred to serum-free medium at T=O. The 24h cumulative incorporation of ^{3}H-dThd was then determined for three sequential periods from T=O to 24h, 24h to 48h, and 48h to 72h respectively. As seen in Table 1, the density of radiolabelled nuclei observed (1 uCi/5ml, 5 Ci/mmole ^{3}H-dThd) during the first 24h period following serum withdrawal was found to be 2.23 x 10^{3} labeled nuclei/cm^{2}, whereas, in those treated with 10^{-8}M TPA this value increased to 6.13 x 10^{3} reflecting the expected mitogenic activity. The number of labeled nuclei observed in the second and third 24h period was reduced to 0.52 and 0.88 labeled nuclei/cm^{2} as the cells reached quiescence. The total cell density showed a similar reduction from 8.38 x 10^{4} nuclei/cm^{2}, to a stable plateau of 5.9 x 10^{4} nuclei/cm^{2} at the end of the third and final 24h period. When TPA was added during the second or third periods after serum

TABLE 1: TPA Effects on C3H-10T1/2 Cells After Serum Reduction

Period of TPA/3H-dThd Addition	Cumulative Nuclear Labeling 10^3 Labeled Nuclei/sq.cm.		10^4 Viable Cells/sq.cm.	
	-TPA	+TPA	-TPA	+TPA
0 - 24h	2.23 ±0.03	6.13 ±0.66	8.38	15.18
24 - 48h	0.52 ±0.01	7.80 ±0.52	6.24	11.54
48 - 72h	0.88 ±0.02	5.97 ±0.70	5.85	5.72

(*) Cultures were seeded at 10^4 cells/60mm plate and allowed to proliferate until approximately 10^5 cells/plate (BME + 10% fetal bovine serum). Medium was then replaced with BME containing 0.5% serum and 0.1 uM TPA + ^{3}H-dThd (1 uCi/plate) added at 0, 24, and 72 h. After 24h, each plate was fixed and prepared for autoradiographic analysis. The number of labeled and non-labeled nuclei was determined using an electronic image analysis system (Artek, Inc.) attached to a Nikon inverted phase contrast microscope. Each field corresponded to $6x10^{-3}$ sq.cm. and the results expressed in terms of the mean ± standard error, where n = 100 fields in duplicate culture plates.

withdrawal, note that the labeled nuclear density did not change substantially even though the total cell density had progressively diminished from 15.2 x 10^4 at 24h, to 11.5 x 10^4 at 48h, and finally to 5.7 x 10^4 at 72h.

This data indicates that there are two cell responses to TPA which are probably independent. First, TPA induces a mitogenic response which is not influenced by the length of time following serum withdrawal. However, TPA also increased cell attachment, and the relative degree of enhanced attachment progressively diminished over the 72h length of the experiment until no enhancement was found in the final 24h period of treatment. As previously described, released cells collected after serum deprivation exhibited extensive DNA degradation (12). Therefore, it was likely that two populations of cells were generated by the stress of serum removal, one responsive to the mitogenic activity of TPA, the other responsive to TPA induced enhanced adhesion. This latter population was apparently also in the process of degeneration and death, a process which reached completion by approximately 72h after serum removal.

It should be noted here that initiation of DNA fragmentation, as well as the inhibition by TPA, were not influenced when experiments were performed under anaerobic conditions (data not shown). Therefore, it is unlikely that molecular oxygen species are directly associated with this phenomenon.

High Specific Activity ^{3}H-dThd Incorporation: In order to induce cell death in a more controlled manner, replicate cultures in exponential proliferation were treated with ^{3}H-dThd at 80 Ci/mmole (1uCi/ml). The time course of DNA fragmentation was then followed in the presence and absence of DHTB (10^{-8}M) as shown in Figure 1. Consistent with the kinetics of cell death following serum withdrawal, DNA fragmentation progressed linearly and approached completion at 96h after ^{3}H-dThd addition. The presence of DHTB during this period inhibited fragmentation by approximately 74%.

As shown in Table 2, the degree of DNA fragmentation was not simply related to the total ^{3}H-dThd incorporated into each cell. Note that the TPA-related reduction in DNA fragmentation was actually accompanied by a marked increase in the total amount of radioactivity incorporated into cellular DNA. Thus, the inhibition of fragmentation is clearly not a result of lower final specific activities of ^{3}H within the DNA. Autoradiographic evidence (not shown) indicated that more than 95% of the cell nuclei were heavily radiolabelled at 48h after high specific activity ^{3}H-dThd addition.

Since apoptosis is a process dependent upon expression of a suicide gene product(s), it was necessary to ensure that the induction of DNA fragmentation was also sensitive to low concentrations of cycloheximide. Also presented in Table 2 are comparisons of TPA treatment with that of cycloheximide (10^{-5}M), and a combination of TPA and cycloheximide. The results indicated that cycloheximide markedly inhibited DNA fragmentation as

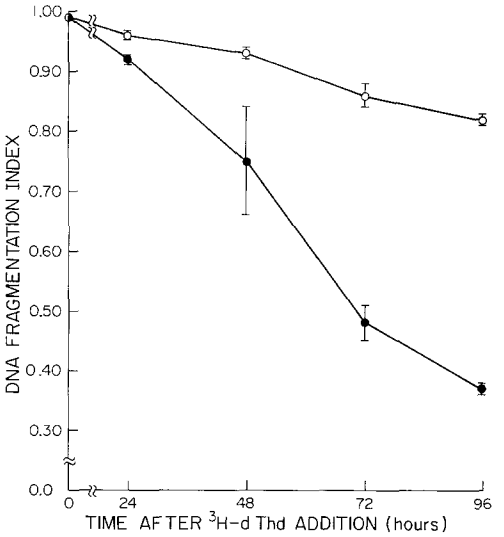

Figure 1:
The time course of DNA fragmentation following exposure of exponentially proliferating cells to 80 + Ci/mmole tritiated thymidine. The closed circles are the Solvent Control cultures and the open circles are the cultures treated with 10 nM dihydro-teleocidin B at the time of thymidine addition.

Table 2: The Effect of TPA and Cycloheximide on DNA Fragmentation in Replicate cultures of C3H 10T1/2 Cells Following Incorporation of High Specific Activity ^{3}H-dThd (>80 Ci/mM)

Hours	Solvent Control		TPA (10^{-7}M)		Cyclohex. (10^{-5}M)		TPA + Cyclohex	
	CPM[1] (X10^{4})	F[2]	CPM[1] (X10^{4})	F[2]	CPM[1] (X10^{4})	F[2]	CPM[1] (X10^{4})	F[2]
48	13.3	0.90	12.6	0.89	nd	0.92	11.3	0.87
72	31.4 (±4.4)	0.54 (±0.04)	63.9 (±30.5)	0.78 (±0.04)	11.0 (±2.2)	0.86 (±.05)	12.6 (±2.4)	0.88 (±0.03)
96	14.9 (±12.4)	0.56 (±.06)	75.2 (±7.8)	0.78 (±.05)	10.6 (±2.0)	0.82 (±.07)	18.3 (±0.9)	0.83 (±.07)

[1] Total cumulative incorporation of high specific activity ^{3}H-dThd (± standard deviation, n=4). Exponentially proliferating cultures received 2 uCi ^{3}H-dThd with specific activity of 80-90 Ci/mM. At 48, 72, and 96h, each culture was rinsed with HBSS before addition of cold 1.5% perchloric acid. Tritium levels were measured in acid insoluble material collected from each culture dish as described previously (17).

[2] F is the DNA fragmentation factor calculated as described in Methods section (12).

did TPA. It is noteworthy that cycloheximide in combination with TPA completely blocked the mitogenic response of TPA.

Gamma Radiation: Precise quantitation of radiation dose is not possible with ^{3}H-dThd incorporation into DNA of cells proliferating in vitro. Therefore, we were interested in examining the effects of TPA on gamma radiation induced cell death. Replicate cultures (n=4) of exponentially proliferating cells were irradiated with 10, 20, 30, and 40 Gy in the presence and absence of TPA (10^{-8}M), and the medium renewed with fresh medium containing ^{3}H-dThd (2 uCi/5ml, 5 Ci/mmole). After 48h, the total cumulative incorporation of ^{3}H was determined and the results presented in Figure 2.

In the solvent treated control cultures, graded doses of radiation did not result in significant changes in the total amount of ^{3}H-dThd incorporated over 48h. However, it was noted that cell morphology degenerated substantially during this period as shown in Figure 3. However, a quite different response was noted in the TPA treated cultures. The graded dose of radiation produced a dose dependent depression in the enhanced mitogenic response due to TPA until the response at 40 Gy was not significantly different from that of the solvent treated cultures which received fresh

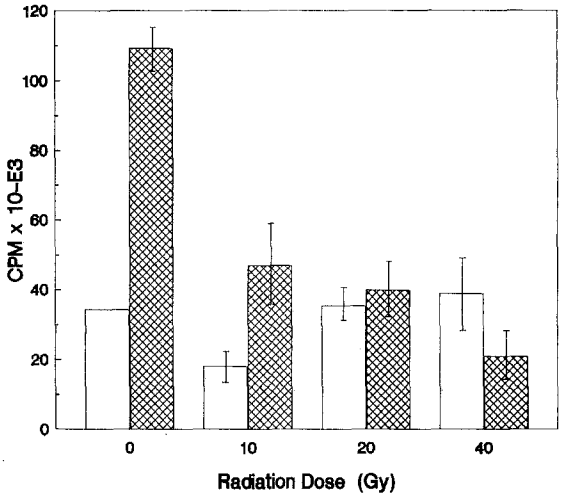

Figure 2:
The effect of graded radiation doses on TPA stimulation of 48h cumulative incorporation of ^{3}H-dThd (2 uCi/5ml culture, 5 Ci/mmole). Replicate cultures (n=4) were irradiated and immediately transferred to fresh medium with and without 10^{-6}M TPA. Solvent controls (open), and TPA (shaded) values are expressed in terms of CPM/replicate culture $\pm$ standard deviation.

medium alone. The enhancement of ^{3}H-dThd incorporation by TPA is sensitive to the dose of radiation. However, the effect of graded radiation on morphology of these cells was radically different in TPA treated cells from that of solvent treated; TPA treated cells showed little change in morphology (Figure 3). All cultures that were maintained for an additional 72h showed a loss of growth activity and no proliferation was sustained following radiation doses above 10 Gy in both solvent and TPA treated groups. However, solvent treated cultures exhibited complete cell degeneration and detachment by the end of the period, whereas, the TPA treated cultures retained a uniform morphology which was not distinct from that of the non-irradiated cultures of TPA treated 10T1/2 cells.

DISCUSSION

These results indicate that tumor promoting phorbol esters inhibit the process of programmed cell suicide which may be initiated in a population following cytotoxic stress. We have examined several modes of cytotoxic stress including heat and chemical carcinogen exposure and presented here data regarding two, acute serum withdrawal, and radiation. We have concluded that tumor promoters afford protection against the cytotoxic and lethal effects of low energy *beta* emission (Table 2, Figure 1), as well as high energy gamma radiation (Figures 2 and 3). To our knowledge, there have been no previous reports of radioprotective activity by tumor promoting agents.

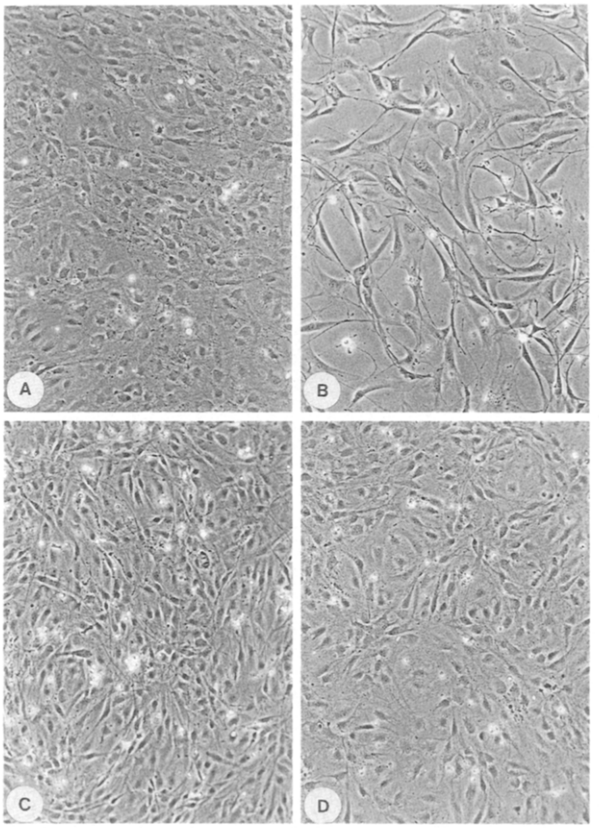

Figure 3:
Photomicrographs of C3H-10T1/2 cells 48h after irradiation and transfer to fresh growth medium; (A,B) solvent control cultures, (C,D) treated with 10^{-8}M TPA. Graded doses of radiation were given at 1.5 Gy/min; shown are cultures that received 0 Gy (A,C), and 40 Gy (B,D). (225X final magnification)

The implications of this data are manifold since it has been shown recently that the process of apoptosis may be critical to a wide variety of biological phenomenon (1-5). Sklar (22) has presented evidence that transformation with *ras* oncogenes enhanced radioresistance of NIH 3T3 cells. In view of the fact that radiation induced cell loss is associated with apoptosis, it is possible that enhancement of radioresistance is mediated by inhibition of apoptosis by oncogene expression. It is also clear that specific radiosensitivity is not necessarily a stable phenotype and that it can be modulated in normal cells. Recently, we reported that TPA profoundly influenced the apoptosis response of human peripheral leukocytes to gamma irradiation (23,24).

These data would support the concept that the inhibition of apoptosis is directly linked to the mechanism of tumor promotion. We feel that this is especially relevant to viral and radiation induced tumorigenesis. Clouston and

Kerr (1) have proposed that lymphocytotoxicity and containment of viral infections are dependent upon the normal physiological function of programmed cell suicide. Furthermore, Valerie *et al.* (26) recently reported that radiation-induced cell suicide is closely associated with concomitant enhancement of HIV replication in infected human cells. Therefore, we feel that it is important to consider the fact that pharmacological modulation of apoptosis may be possible both *in vitro* and *in vivo*. This accessibility may provide the necessary tools to further explore the biological roles of apoptosis, and the ability of cells to initiate self-destruction.

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