INHIBITION OF THYMINE DIMER EXCISION BY THE PHORBOL ESTER, PHORBOL MYRISTATE ACETATE*

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Received November 6,1972

Summary The effect of the promoting agent, phorbol myristate acetate, on repair of UV-induced damage in HeLa cells was studied. The agent decreased survival and subsequent colony-forming ability of irradiated cells and inhibited removal of UV-induced thymine-containing dimers from DNA of irradiated cells.

Introduction: Cocarcinogenic agents are substances which promote the development of tumors in animals previously exposed to carcinogens, but are in themselves only weakly tumorigenic (1). It has been demonstrated that phorbol esters, the active factors purified from the promoting agent, croton oil, and other cocarcinogenic agents, inhibit UV-induced unscheduled DNA synthesis in human lymphocytes (2,3). Therefore, it has been proposed that inhibition of DNA repair is a mechanism of action of promoting agents (2,3).

The relationship of defective DNA repair to carcinogenesis has been made apparent by the finding that cells of patients with Xeroderma Pigmentosum (XP), a hereditary skin disease characterized by cutaneous photosensitivity and a high incidence of cutaneous malignancies, demonstrate virtual absence of repair replication and unscheduled DNA synthesis after UV irradiation. Because these cells are unable to excise thymine-containing dimers from their irradiated DNA, the defect in the disease is thought to be at the level of the UV specific endonuclease (5). The relevance of this defect to carcinogenesis was made even more significant by the finding that XP cells are unable to repair the damage to DNA caused by the carcinogen N-acetoxy-2-acetylaminofluorene (6).

* Supported by grant number 5 R01 CA10978-05 from the National Cancer Institute.

In order to elucidate the mechanism by which promoting agents inhibit DNA repair, we chose to investigate whether a phorbol ester with strong promoting ability, phorbol myristate acetate (PMA), would specifically inhibit thymine dimer excision in irradiated cells and whether the agent would have an effect on survival and colony formation of irradiated cells grown in culture.

Materials and Methods:

Survival Studies: HeLa cells were grown in plastic dishes in Dulbecco's Modified Eagle Medium (DME) supplemented with 10% calf serum. The irradiation source was a 15 watt germicidal lamp (GE G15T8). Cells were irradiated at a dose rate of 6 ergs/mm^2/sec. in phosphate-buffered saline. After irradiation they were immediately trypsinized and plated in Linbro 16mm dishes (Linbro Chemical Co., Inc., New Haven, Conn.) in DME in the presence or absence of PMA. One mg of PMA was dissolved in one ml of anhydrous ethanol and added to medium to a final concentration of 10^{-3} mg/ml. An equivalent amount of ethanol was added to control cultures. Colonies were counted 7-10 days after irradiation. Four plates per point were counted, the result averaged and expressed as percent survival. Results were plotted on semi-log paper, the lines drawn by eye and the D_0 estimated from the exponential portion of the plot. Plating efficiency was 85-90% in the presence or absence of PMA and this was taken as 100% survival.

Dimer Excision: Cells were grown under conditions similar to above. They were incubated for 24 hours prior to irradiation with H^3-thymidine (New England Nuclear) at a concentration of 1.25 microcuries/ml and unlabelled thymidine to a final nucleoside concentration of 0.7 micrograms/ml. Two hours before irradiation the medium was removed and replaced with unlabelled medium. The cells were irradiated to total doses of 80 and 160 ergs/mm^2, harvested either immediately after irradiation or allowed to repair for 24 hours in the presence or absence of PMA (10^{-3} mg/ml). A nuclear preparation was made by the method of Penman (7). The nuclear pellet was resuspended and precipitated with cold 5% trichloroacetic acid. After repeated acid and ethanol washes, the

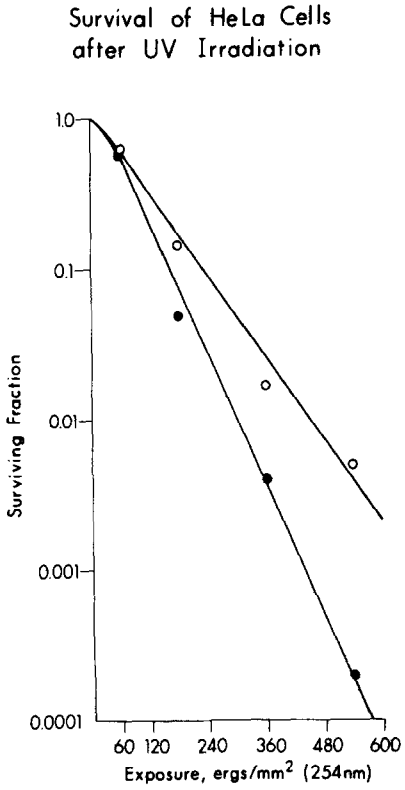

Figure 1. Survival of HeLa cells after UV irradiation. Open circles represent control cultures. Closed circles represent PMA cultures. Each point represents the average of four plates. The lines are fitted by eye.

pellet was hydrolysed in formic acid and dimer content determined by the method of Carrier and Setlow (8), using a Nuclear Chicago Mark II liquid scintillation counter. Three to 5 determinations were done for each experimental point and the averages and standard errors calculated.

Results and Discussion: Figure 1 demonstrates the altered survival of HeLa cells grown in the presence of PMA after irradiation. D_0 for control cultures was estimated to be 96 ergs/mm^2, a figure which agrees with the previously reported value (9). D_0 for cells grown in the presence of PMA was 60 ergs/mm^2. A shoulder seems to be present in both plots and survival at the low dose of UV (60 ergs/mm^2) was the same, indicating that at low doses of irradiation, PMA had no apparent effect. This is corroborated by the finding

UV Dose (Ergs/mm^2)	% Dimers		
	0 Time	24 Hours	
		Control	PMA
80	.031 ± .002	.015 ± .001	.015 ± .001
160	.053 ± .002	.034 ± .002	.050 ± .004

Table 1. Release of thymine-containing dimers from DNA of irradiated HeLa cells. Values represent the average of three to five separate determinations ± standard error of the mean.

that dimer excision was not inhibited at 80 ergs/mm^2 (Table 1). Diminished survival was manifest at higher doses of UV and at 160 ergs/mm^2, a region in which cell survival was 5 times greater in control cultures than in PMA, dimer excision was completely inhibited.

These results indicate that PMA inhibits the endonuclease-associated step of UV repair in HeLa cells. That this is not merely a toxic effect is indicated by the fact that PMA had no effect on plating efficiency of cells.

If the endonuclease step is the only phase of DNA repair inhibited by PMA, one would expect survival of HeLa cells after ionizing radiation not to be altered in the presence of PMA. This situation would be analogous to XP cells which apparently repair damage caused by ionizing radiation normally (10). On the other hand, if survival were to be altered in HeLa cells exposed to ionizing radiation in the presence of PMA, this would suggest that the agent affects other steps of DNA repair in addition to the endonuclease step. Experiments are currently in progress to determine the effect of PMA on survival of HeLa cells after ionizing radiation.

Acknowledgement: We thank Drs. Benjamin Van Duuren and Alvin Segal for generously supplying PMA. The technical assistance of Mr. Archie Cummings is appreciated.

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