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EPIDERMAL GROWTH FACTOR AND TUMOR PROMOTERS PREVENT DNA FRAGMENTATION BY DIFFERENT MECHANISMS

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Serum deprivation of C3H 10T 1/2 fibroblasts resulted in DNA fragmentation which was prevented by growth factors such as Epidermal Growth Factor or the tumor promoters, 12-0-tetradecanoyl-13-0-phorbol acetate and Dihydroteleocidin B. Palmityl carnitine, an inhibitor of Ca2+-phospholipid-dependent protein kinase C, reversed the effects of the tumor promoters, but not the effect of Epidermal Growth Factor.

A number of cytotoxic effectors including X-irradiation, alkylating agents and chemotherapeutic drugs such as Adriamycin have been reported to induce site specific DNA damage suggestive of endonuclease activation (1,2). We have also observed genomic breakdown of mouse embryonic fibroblast cultures deprived of serum for 24 h or more. The mechanism by which subpopulations of cells with highly fragmented DNA arise is not understood, and the present study is concerned with the findings that growth factors and tumor promoters prevent DNA fragmentation. One of the purposes of this manuscript is to draw attention to potential problems involving the interpretation of results of experiments performed under similar conditions of serum deprivation which is widely used to stage cells in G_1.

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ABBREVIATIONS: TPA, 12-0-tetradecanoyl phorbol-13-acetate; [^{3}H]-dThd, Tritiated thymidine; EGF, Epidermal growth factor; DHTB, Dihydroteleocidin B; FDGF, Fibroblast derived growth factor

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MATERIALS AND METHODS

Cell-Cultures : Cultures of C3H 10T 1/2 (clone 8) were used between the 7th and 14th series passage. Cultures were maintained as previously described (3,4) at 37 with 5% CO_2 in Eagles Basal Medium (BME) supplemented with 10% heat-inactivated fetal bovine serum.

Alkaline sucrose gradients : The extent of DNA fragmentation was estimated by distribution of radioactivity in linear alkaline sucrose gradients in both adherent and non-adherent cells 24 h after serum deprivation. Linear alkaline sucrose gradients (5-20%; 11.6 ml) were prepared in 0.3 N NaOH, 0.7 M NaCl, and 0.01 M Na_4EDTA. C3H-10T 1/2 cells were prelabeled for 24 hr with [3H]-dThd (0.2 Ci/ml, 4-6 Ci/m mole,) prior to serum withdrawal. After 24 h in serum free medium with and without agents, the cells were dispersed, washed with medium, and concentration adjusted to 5 X 10^4 cells/0.2 ml. The cells were lysed overnight after slow addition to a solution (0.2 ml) containing 1.0 N NaOH, 0.1 M NaCl, and 0.01 M Na_4 EDTA overlying the gradient. The tubes were centrifuged (Beckman L2-65B) in a SW41 rotor at 30,000 RPM for 150 min (1). Fractions (8 drop) collected from the top of the tubes by a Densi-Flow II apparatus (Searle, Fort Lee, NJ) were acidified by addition of 1.0 N HCl (0.5 ml), and counted by standard liquid scintillation technique.

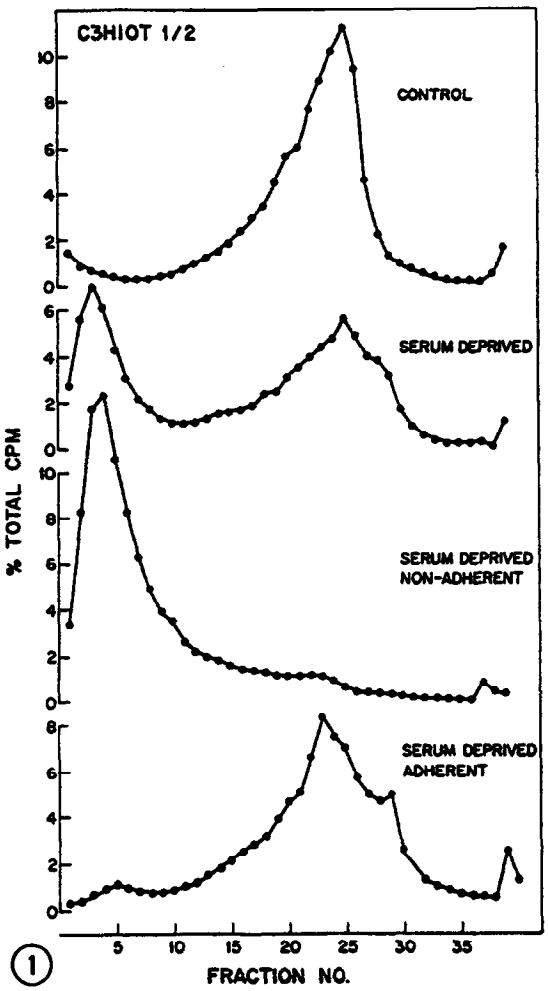
Agents : 12-0-tetradecanoyl phorbol-13-acetate (TPA) was obtained from Dr. Peter Borchert (Eden Prairie, WI), dihydroteleocidin B (DHTB) from Drs. H. Fujiki and T. Sugimura (Tokyo, Japan). Each agent was added in 10 µl aliquots (5 ml total culture volume), whereas all controls received 10 µl of solvent alone. Insulin (bovine) at 26.1 USP insulin units/mg was obtained from GIBCO, Grand Island, NY. Purified EGF was a gift from Dr. Richard Savage (Temple U., Phila., PA).

RESULTS

Linear alkaline sucrose density gradient analysis of DNA from cells released by serum deprivation and adherent cells :

Initially, prelabeled cultures of exponentially grown C3H 10T 1/2 cells were washed and fed with serum-free medium with. After 24 h, non-adherent cells were separated from adherent cells by decantation, and analyzed on alkaline sucrose density gradients. The results (Fig. 1) indicate that serum deprivation leads to a non-random pattern of DNA damage in C3H 10T 1/2 cells, consisting of a population of small DNA fragments (near top of gradient), and DNA of a size similar to control DNA. When the subpopulation of adherent and nonadherent cells were separated and lysed separately, it was apparent that the non-adherent cell population contained the highly fragmented DNA, while the adherent cells contained DNA of high molecular weight.

Effect of TPA on serum-induced DNA fragmentation It is apparent that soon after removal of serum, a subpopulation emerges as



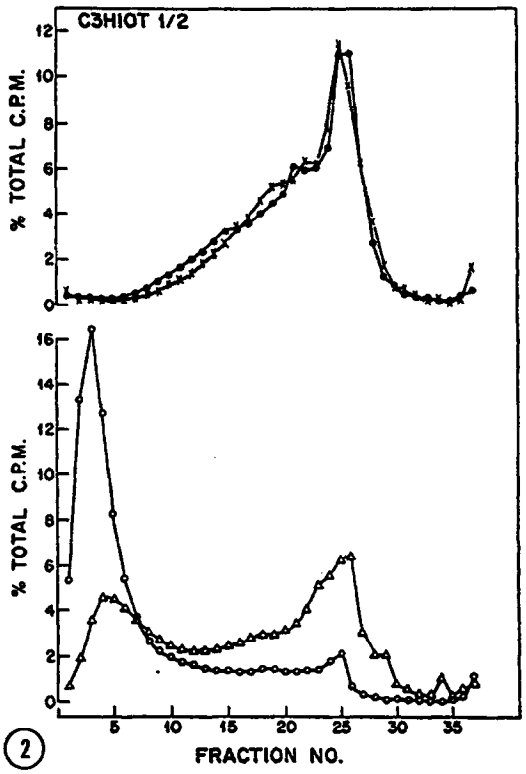


Figure 1: Effect of serum deprivation on sedimentation of C3H10T 1/2 cellular DNA in linear alkaline sucrose gradients. Prelabeled (24h, 0.2 uCi/ml media) cells were washed and fresh media with or without serum was added. They were then reincubated (37°C) for 24h. Cells were removed from plates mechanically (rubber policeman), collected by centrifugation, and DNA size distributions determined by sedimentation in linear (5-20%) alkaline sucrose gradients (fraction 1 is at the top of the gradient). The uppermost panel shows the sedimentation profile of control (non-serum deprived) cells; the panel below is from the total cell population of a serum deprived culture; and the two lower panels show the separated adherent and non-adherent subpopulations from a serum deprived culture.

Figure 2: Effect of serum deprivation and TPA on sedimentation of C3H10T 1/2 cellular DNA in linear alkaline sucrose gradients. Prelabeled (24h, 0.2 uCi/ml [^{3}H]-Thd) cells were washed, fresh media with serum (-○-), media with serum and TPA (3.3×10^{-5}M) (-X-), media without serum (-●-), and media with TPA (3.3×10^{-7}M) without serum (-△-) were added, reincubated for 24h, and sedimentation analysis of DNA size determined as outlined in Figure 1.

non-adherent cells that have extensive specific DNA damage. These cells remained viable for 24 to 72 h, (greater than 95% viability by trypan blue exclusion). It was then of interest to compare cells

incubated in serum-free medium with 10^{-7}M TPA. In the presence of TPA, the non-adherent viable cells reattached and spread on the culture plate surface. As shown in Fig. 2, TPA did not induce any change in the DNA profile in the presence of serum. However, when TPA was added at the time of serum deprivation, DNA fragmentation was markedly reduced (25-50%). The inhibition of DNA fragmentation correlated with induction of cell adhesion by tumor promoting phorbol esters. It is believed that the reattached cells represent a G_1 state previously described as TPA resistant (4).

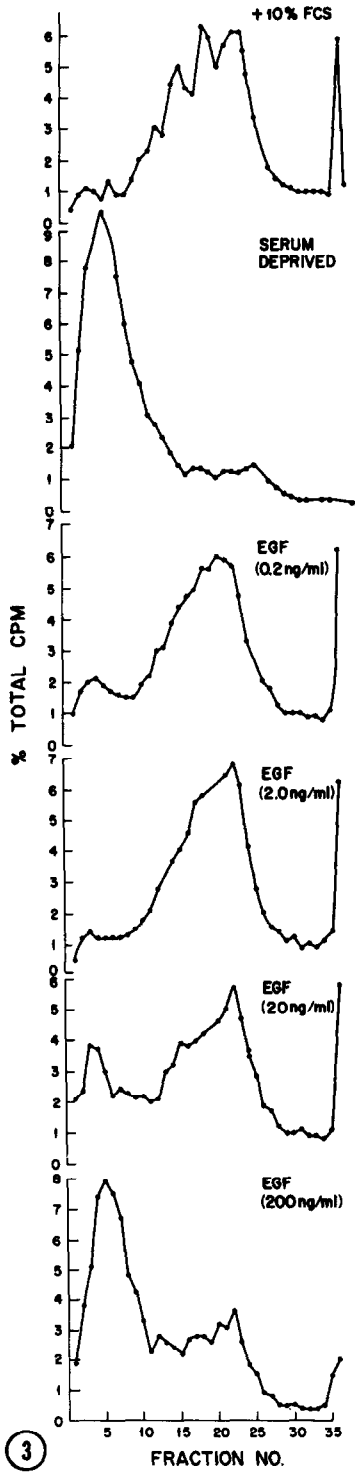
Growth factor effects on DNA fragmentation:Biphasic responses of EGF

The ability of growth stimulants such as EGF and insulin to inhibit DNA fragmentation was examined next. Insulin at 1.0ug/ml failed to provide any protection of DNA fragmentation induced by serum deprivation despite its ability to stimulate glucose utilization (data not shown). However, EGF at concentrations as low as 0.2 ng/ml almost completely prevented DNA fragmentation induced by serum removal (Fig. 3). There was no difference in the protection against fragmentation afforded by EGF at 2.0 ng/ml from that obtained with 10% fetal calf serum. However, as the concentration of EGF was raised there was a diminished response such that at 200 ng/ml EGF, DNA fragmentation approached that obtained in BME alone.

It should be pointed out that ouabain, an inhibitor of serum or tumor promoter-induced DNA synthesis, did not alter protective effects induced by EGF or TPA. These findings indicate that $(Na^+ + K^+)$-ATPase is not involved in the mechanism by which DNA breakdown is prevented by these mitogenic agents.

Reversal of protective effects of tumor promoters but not EGF by palmityl carnitine

Palmityl carnitine has been reported to block the activity of protein kinase C (5), the presumed receptor of diterpene tumor



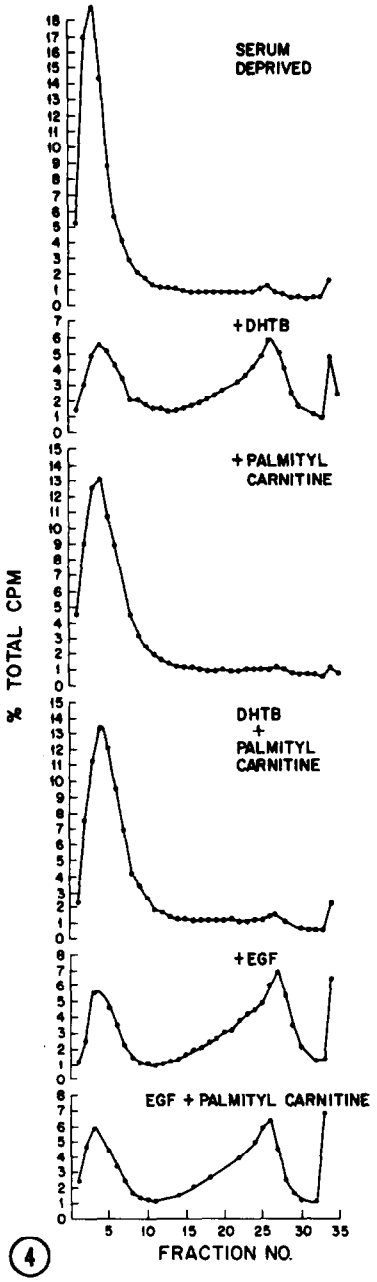


Figure 3: Effect of EGF concentration on serum deprivation induced DNA fragmentation. Prelabeled post-confluent C3H10T 1/2 cells were serum-deprived for 48h in BME in the absence and presence of 10% fetal calf serum or EGF at concentrations as designated.

Figure 4: Effect of palmityl carnitine on the ability of DHTB

promoting agents. In Figure 4, the ability of palmityl carnitine to reverse the protective effect induced by the potent tumor promoter (DHTB), is examined. It can be seen that the protective effect afforded by DHTB at concentrations as low as 10^{-8} M is diminished when palmityl carnitine is also present. This finding suggests that the protective effect of DHTB is mediated by activation of protein kinase C. In contrast, palmityl carnitine did not alter the ability of EGF to prevent DNA fragmentation, indicative that its protective action is not mediated by protein kinase C.

DISCUSSION

The present report indicates that serum deprivation results in release of cells that have a uniformly fragmented DNA, complete repair of which is unlikely. The cells released by serum deprivation survive approximately 72 h unless EGF, epidermal growth factor, serum or tumor promoters such as TPA or DHTB are added, and these cells remain part of the adherent fraction where no such decline in viability is evident and fragmentation is observed to be markedly reduced.

The finding that epidermal growth factor protects DNA breakdown as effectively as fetal calf serum complements previous data which indicates that growth factors such as EGF, FDGF, etc, stimulate DNA synthesis in several cell types (6-8). Ouabain, an inhibitor of the mitogenic action of serum and of other growth factors as well as of tumor promoters, did not counteract the protective effects of serum-induced non-random DNA fragmentation. Thus, it does not appear likely that Na+ or K+ movements mediated by (Na+ + K+)-ATPase activity are involved in the protective action.

Although insulin has also been reported to stimulate growth in some cell types (9), the present studies indicate that insulin does not

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and EGF to prevent DNA breakdown. Prelabeled post-confluent C3H10T 1/2 cells were serum deprived for 48h in BME in the presence or absence at 10^{-8}M DHTB or EGF $\pm$ palmityl carnitine (10^{-5}).

prevent fragmentation of DNA. EGF and tumor promoters induce responses by a different mechanism. Palmityl carnitine, which has been reported to inhibit protein kinase C, reversed the protective effect induced by tumor promoters such as DHTB or TPA but did not affect the protective action afforded by EGF. In view of the failure of TPA or DHTB to prevent DNA breakdown when palmityl carnitine was added, it is assumed that activation of protein kinase C by the tumor promoters is intimately involved in the protective responses elicited by these agents. It is also reasonable to expect that the EGF protective mechanism involves phosphorylation by its respective kinase. Whether these agents share a common site for the eventual protective action by which DNA fragmentation is minimized, is not clear.

Previous studies have indicated that growth stimulating activity of tumor promoters such as TPA is dependent on the presence of serum factors (cf 10). However, the present studies suggest that the failure to observe mitogenic effects with TPA or DHTB alone may be related to serum-deprived conditions in which DNA fragmentation occurs to a degree that replication is no longer possible.

It is, also interesting to speculate that tumor promotion may be intimately related to the facilitation of DNA damage repair (or inhibition of a generalized breakdown of the genome) sufficiently so as to allow replication of even a small proportion of affected cells. Non-repair associated DNA fragmentation may result in decreased cell survival, and tumor promoters may enhance tumorigenesis by prevention of endonuclease-associated genomic breakdown which leads to cell death. Our conclusion that endonuclease activities are involved in serum-deprived DNA fragmentation is based on the non-random fragmentation pattern as indicated by the sharp peaks obtained in gradient studies.

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