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INHIBITION OF REPLICON INITIATION BY 12-O-TETRADECANOYLPHORBOL-13-ACETATE

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SUMMARY: To investigate the inhibition of DNA replication by tumor promoters, we incubated HeLa cells with 12-O-tetradecanoylphorbol-13-acetate (TPA; 10^{-8} to 10^{-5} g/ml) and quantified DNA synthesis on alkaline sucrose gradients. TPA was found to selectively inhibit replicon initiation without affecting DNA chain elongation in replicons that had already initiated. No inhibition of DNA synthesis was seen when cells were exposed to the nonpromoting derivative of TPA, 4- α-phorbol 12,13-didecanoate. Superoxide dismutase did not prevent the TPA-induced inhibition of initiation.

The tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), stimulates the development of latent tumor cells *in vivo* (1,2) and the transformation of cells *in vitro* (3,4). TPA produces a wide range of responses in cells (2), including alterations in cyclic nucleotide concentrations; changes in cell membrane function; inhibition of DNA replication followed by increased DNA, RNA, and protein synthesis; and increased histone phosphorylation. TPA is not carcinogenic or mutagenic on its own (2,5) and is therefore not considered to be a DNA-damaging agent.

Kinzel et al. (6) recently reported that in HeLa cells 10^{-7} M TPA produced a transient block in G_2 as well as inhibiting DNA synthesis. In these characteristics, TPA

Abbreviations: 4- α-PDD, 4- α-phorbol 12,13-didecanoate; SOD, superoxide dismutase; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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resembles X rays (7). The inhibition of DNA replication produced by low doses of X rays (<10 Gy) is the consequence of a specific reduction in the rate of replicon initiation (8,9). We examined the effects of TPA on replicon initiation in HeLa cells to determine if TPA is similar to X rays in this respect as well.

TPA has been shown to induce sister chromatid exchanges in Chinese hamster, mouse, and human cell lines (10-13), although this finding is controversial (14-17). Nagasawa and Little (18) have reported that the induction of sister chromatid exchanges by TPA in hamster cells was inhibited by superoxide dismutase (SOD), a scavenger of superoxide anion radicals. Therefore, we examined the effects of SOD on the inhibition of DNA synthesis induced by TPA to determine if free radicals mediate this phenomenon.

MATERIALS AND METHODS

HeLa cells were grown in Eagle's minimal essential medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin under a water-saturated atmosphere of 5% CO_2. Replicate cultures were prepared by seeding cells into 60 mm plastic petri dishes (3.5×10^3 cells/cm^2) in medium containing 0.01 μCi/ml [^{14}C]thymidine (spec. act., 50 Ci/mol). After two days, the ^{14}C-labeled medium was replaced with fresh unlabeled medium, and treatments followed two or more hours later. TPA (a gift of Dr. J.A. Miller), made up in a stock solution of 10^{-4} g/ml in ethanol, was added to the medium to a final concentration of 10^{-10} to 10^{-5} g/ml. [^{3}H]thymidine (spec. act., 80 Ci/mmol) was added to a final concentration of 10 μCi/ml at various times after the addition of TPA, 4-α -phorbol 12,13-didecanoate (4-α -PDD, 10^{-8} g/ml), a nonpromoting phorbol ester (19), or ethanol (0.5%). In some experiments, SOD (1450 units/ml) was added alone or in combination with TPA (10^{-8} g/ml) 30 min before the addition of [^{3}H]thymidine. After 10 min incubation with [^{3}H]thymidine at 37° C, the medium was quickly drained and the cells were rinsed with ice-cold SSC (0.15 M sodium chloride, 0.015 M sodium citrate). Cells were harvested and radioactive DNA strand sizes were determined in alkaline sucrose gradients as previously described (9,20).

Table I. Inhibition of DNA Replication by TPA

Concentration of TPA (g/ml)	Percent of control DNA synthesis in replication intermediates with molecular weights of		
	$14\text{-}23 \times 10^7$	$5\text{-}10 \times 10^7$	$0.7\text{-}2.5 \times 10^7$
10^{-10}	109	103	100
10^{-8}	95	94	72
10^{-6}	98 ± 13*	96 ± 7*	67 ± 7*
10^{-5}	92	90	59

* Mean ± s.d., n = 7.

HeLa cells were incubated with [^{3}H]thymidine for 10 min beginning 30 min after addition of TPA or ethanol. [^{3}H]DNA strands labeled during the pulse were separated by sedimentation through 5-20% alkaline sucrose gradients, and the ^{3}H cpm recovered in each gradient fraction were normalized to a constant number of cells. Normalized ^{3}H radioactivity was summed for gradient fractions 7-10, 12-15, and 17-20 (Fig. 1), which contained DNA with molecular weights of $14\text{-}23 \times 10^7$, $5\text{-}10 \times 10^7$, and $0.7\text{-}2.5 \times 10^7$ daltons, respectively. Values obtained for TPA-treated cells are expressed as a percentage of the value obtained in the ethanol-treated controls.

RESULTS

Thirty min after the addition of TPA to culture medium at concentrations ranging from 10^{-8} to 10^{-5} g/ml, DNA synthesis in replication intermediates of 0.7 to 2.5 x 10^7 daltons was inhibited by 28 to 41% whereas synthesis in larger intermediates was not affected (Table I, Figs. 1 and 2). The lower concentration of 10^{-10} g/ml was without effect. After 30 min, the inhibition produced by 10^{-6} g/ml of TPA spread to include larger intermediates ($5\text{-}10 \times 10^7$ and $14\text{-}23 \times 10^7$ daltons; Fig. 1). This characteristic "sweeping out" of the inhibition of DNA synthesis from low molecular weight to high molecular weight replication intermediates with time after addition of TPA is similar to

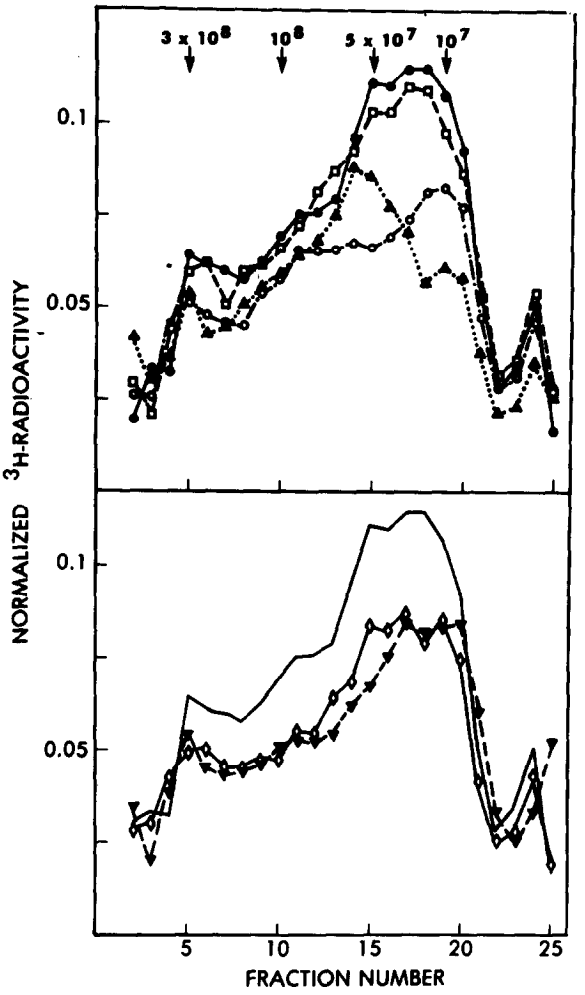

Fig. 1. Time course of inhibition of DNA synthesis in HeLa cells by TPA. Cells were incubated for 10 min with [^{3}H]thymidine beginning 0 (□), 30 (▲), 60 (○), 90 (▼), or 120 (◇) min after addition of TPA (10^{-6} g/ml) or 30 min after addition of ethanol (0.5%) (●). After labeling, the cells were chilled and harvested, and the quantity and size distribution of labeled DNA strands were determined by velocity sedimentation in 5-20% alkaline sucrose gradients. The direction of sedimentation is from right to left. Normalized ^{3}H radioactivity represents the net ^{3}H cpm per fraction normalized to the number of cells applied to gradients. The solid line in the bottom panel is a tracing of the control profile from the upper panel.

that seen after low doses of X rays (9) and is indicative of a selective inhibition of replicon initiation. Even at a

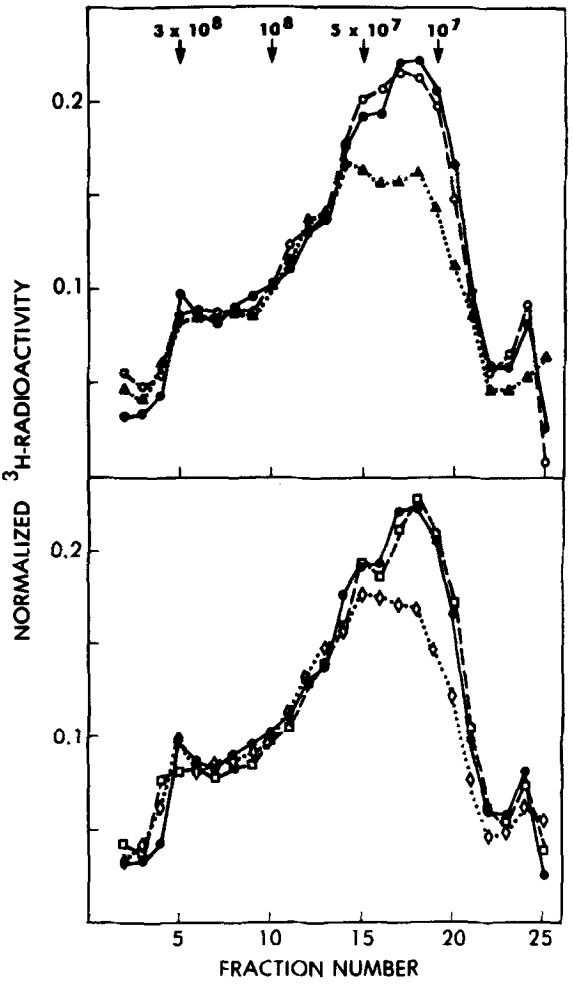

Fig. 2. Effect of phorbol esters and superoxide dismutase on DNA synthesis in HeLa cells. (Top) Cells were incubated for 10 min with [^{3}H]thymidine beginning 30 min after addition of TPA (10^{-8} g/ml) (▲), 4-α-PDD (10^{-8} g/ml) (○), or ethanol (0.5%) (●). (Bottom) Cells were incubated for 10 min with [^{3}H]thymidine beginning 30 min after addition of SOD (1450 units/ml) and ethanol (0.5%) (□), or SOD (1450 units/ml) and TPA (10^{-8} g/ml) (◇), or ethanol (0.5%) (●). After labeling, cells were harvested and the DNA was sedimented in 5-20% alkaline sucrose gradients. ^{3}H radioactivity was normalized as described by the legend to Fig. 1.

concentration of 10^{-5} g/ml, TPA did not appear to affect the rate of DNA chain elongation in replicons already in operation.

The inhibition of replicon initiation was greatest 30

minutes after addition of 10^{-6} g/ml TPA, with partial recovery of initiation evident within 60 min. By 120 min after addition of TPA cells had established a new steady state of replicon initiation and processing, but with an initiation rate about 70% of control.

No inhibition of DNA synthesis was seen after the addition of the nonpromoting phorbol ester, 4- α-PDD (10^{-8} g/ml) to the medium (Fig. 2). Also, the inhibition produced by 10^{-8} g/ml TPA was not affected by the addition of SOD (1450 units/ml; Fig. 2).

DISCUSSION

The mechanism by which X rays inhibit replicon initiation is unknown. The inhibition is believed to be due to an effect on the structure of chromatin mediated through DNA strand breaks (21). The radiomimetic activity of TPA on DNA synthesis also remains unexplained but is probably related to its tumor-promoting activity because 4-α-PDD had no effect on DNA synthesis. Moreover, the effect was apparent with concentrations of TPA that promote cell transformation *in vitro*. The inhibition of initiation does not appear to be mediated through superoxide anion radicals because SOD did not prevent this response.

In the absence of clear understanding, we might speculate that, because TPA indirectly alters chromatin structure (e.g., through histone phosphorylation) its effect on replicon initiation also could be due to TPA-induced changes in chromatin organization. The state of supercoiling of the chromatin fiber has been shown to

influence replicon initiation rate (22,23). It is conceivable that rapid changes in chromosome organization produced by TPA cause a reduction in replicon initiation rate by masking replicon origins or by altering the coiling of the chromatin fiber. It will therefore be of interest to determine whether other inducers or repressors of gene activity affect replicon initiation.

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