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Tumor promoters inhibit spontaneous and induced differentiation of murine erythroleukemia cells in culture

(phorbol esters/Friend virus/differentiation)

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ABSTRACT **Addition of the potent tumor promoter, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), to murine erythroleukemia cell lines in suspension cultures inhibited both spontaneous differentiation and differentiation induced by hexamethylene bisacetamide (HMBA), dimethyl sulfoxide, or butyric acid. Inhibition was unrelated to cytotoxicity and was reversible. When several plant diterpenes were tested, there was a positive correlation between tumor-promoting activity and inhibition of differentiation. TPA inhibited HMBA-induced differentiation only if added prior to the time of commitment to differentiation, as assayed by scoring for differentiation after transfer of cells from HMBA to fresh medium without HMBA. TPA-mediated inhibition of differentiation was associated with a decrease in globin mRNA accumulation.**

A murine erythroleukemia cell (MELC) line derived from that established by Friend *et al.* (1) shows a low level (<0.5%) of spontaneous erythroid differentiation in culture. Addition of various chemicals, including dimethyl sulfoxide (Me_2SO) (1), hexamethylene bisacetamide (HMBA) (2), and butyric acid (3), to the culture medium results in expression of a program of erythroid differentiation that includes characteristic morphological changes (1), accumulation of globin mRNA (4–7), α- and β-globin chain synthesis (8), increased heme synthesis (9), appearance of erythrocyte-specific membrane antigens (10, 11), and loss of the capacity for cell division (1, 12). Clones with a high percentage of spontaneously differentiating cells have also been isolated (13).

The compound 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and related plant diterpenes are extremely potent tumor-promoting agents in the two-stage mouse skin carcinogenesis system (14–17). Although the mechanism of action of TPA is not known, recent studies in cell culture systems suggest that it induces a phenotypic program mimicking that of transformed cells (18, 19). Because of these effects, as well as the evidence that tumor cells display aberrant differentiation, we have examined the action of TPA on cells undergoing a specific program of differentiation. In this paper, we report that TPA and certain congeners are potent reversible inhibitors of both spontaneous differentiation and differentiation induced by HMBA, Me_2SO, and butyric acid in MELC.

MATERIALS AND METHODS

Chemicals. HMBA was synthesized and purified as described (2). TPA was obtained from the National Cancer Institute, Bethesda, MD. Phorbol, phorbol-12,13-didecanoate (PDD), and 4α-phorbol-12,13-didecanoate (4α-PDD) were kindly provided by Sidney Belman. Ingenol dibenzoate and mezereine were generous gifts from M. Kupchan. All tumor promoters were dissolved in acetone or Me_2SO, and the final concentration of acetone or Me_2SO in the culture medium was 0.1%. This concentration of solvent alone has no detectable effect on cell growth or on the accumulation of benzidine-reactive cells.

Cell Culture. MELC, strain 745A, was kindly provided by Charlotte Friend. Our subclone, designated DS19, has been maintained in suspension culture as described (20). Unless otherwise noted, cultures were inoculated at a density of 10^5 cells per ml from 1-day-old cultures and the percentage of hemoglobin-containing cells was determined by the benzidine reaction after 4 and 5 days. Cells of clone 9, a subclone isolated from DS19, were inoculated at a density of 10^3 cells per ml from 3-day-old cultures and the proportion of differentiated cells was determined at day 6. Cell density was determined with a Coulter counter, model ZF.

Benzidine Staining. Cells from liquid suspension cultures were stained for hemoglobin content by depositing the cells on a glass slide (Cytocentrifuge; Shandon Southern Instruments, Inc., Sewickly, PA), fixation in methanol, and staining by the alkaline benzidine-Wright-Giemsa reaction (20); the proportion of orange-stained cells was scored.

Cytoplasmic RNA Extraction and Hybridization with cDNA. Total cytoplasmic RNA of MELC was extracted as described previously (21) with minor modifications. Cells were washed three times in cold saline, resuspended in 1.8 ml of saline, and lysed by addition of 0.2 ml of 10% Nonidet P40 followed by chilling in ice for 5 min. Nuclei were removed by centrifugation at 500 × *g* for 10 min. The supernatant, containing cytoplasmic RNA, was extracted with a mixture containing 10 ml of phenol/chloroform/isoamyl alcohol, 100:100:1 (vol/vol), and 9 ml of a 0.1 M Tris·HCl, pH 9.0/0.1 M NaCl/1% sodium dodecyl sulfate/1 mM EDTA. RNA in the aqueous phase was precipitated with 2 volumes of cold ethanol in the presence of 0.2 M NaCl at −20° for 18 hr and collected by centrifugation. The RNA was twice reprecipitated with cold ethanol and was dissolved finally in H_2O.

Mouse globin 9S mRNA was purified from DBA/2 mouse reticulocyte polysomal RNA by phenol/chloroform/isoamyl alcohol extraction, followed by oligo(dT)-cellulose chromatography (22) and sucrose gradient centrifugation (23). cDNA was prepared by using the purified globin mRNA as template for reverse transcriptase from avian myeloblastosis virus (obtained from G. E. Houts and J. W. Beard, Life Science Laboratories, St. Petersburg, FL) (24). cDNA·RNA hybridization was carried out in 10-μl capillaries containing 2500 cpm of

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Abbreviations: MELC, murine erythroleukemia cells; Me_2SO, dimethyl sulfoxide; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; HMBA, hexamethylene bisacetamide; PPD, phorbol-12,13-didecanoate; 4α-PDD, 4α-phorbol-12,13-didecanoate.

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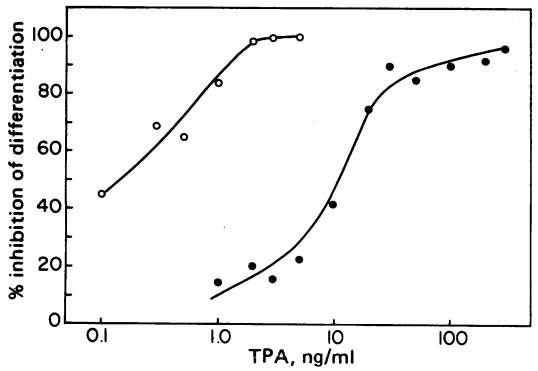

FIG. 1. Effects of TPA on spontaneous and HMBA-induced differentiation of MELC. For studies on spontaneous differentiation (○), clone 9 cells were suspended in growth medium at 10^3 cells per ml and TPA, at the indicated concentrations, was added at time 0. Cultures were incubated at 37° in this medium and scored for benzidine-reactive (B^+) cells on day 6 of culture. For studies on induced differentiation (●), DS19 cells were suspended in growth medium at 10^5 cells per ml. Both HMBA (4 mM) and TPA (at the indicated concentrations) were added at time 0; B^+ cells were scored on day 5. The percentage inhibition is defined as:

$$\frac{[\%\ B^+ \text{ cells, no TPA}] - [\%\ B^+ \text{ cells, with TPA}]}{[\%\ B^+ \text{ cells, no TPA}]} \times 100$$

[^{3}H]cDNA (14,000 cpm/ng) for 24 hr as previously described (25). The relative content of globin-specific RNA sequences was calculated from the amount of total cytoplasmic RNA required for hybridization to 50% of the cDNA in 24 hr.

RESULTS

Effect of TPA on Induced and Spontaneous Differentiation of MELC. MELC (DS19) display less than 1% spontaneous differentiation (benzidine-reactive cells). When cultured with 4 mM HMBA, an increase in the proportion of benzidine-reactive cells is detected at day 2 and reaches a maximum of more than 85% by days 4 or 5 of culture.

Low concentrations of TPA inhibited HMBA-induced differentiation of MELC (DS19) (Fig. 1). Fifty percent inhibition of the effect of 4 mM HMBA was observed with TPA at approximately 12 ng/ml (20 nM). Similar results were obtained with two other inducers of differentiation, Me_2SO and butyric acid. In the presence of Me_2SO, 70–75% of DS19 cells became benzidine-reactive; addition of TPA (30 ng/ml) decreased differentiation to less than 10% (more than 90% inhibition). Butyric acid (1.2 mM) induced differentiation of about 60–70% of DS19 cells. TPA (30 ng/ml) inhibited butyric acid-induced differentiation by approximately 40–50%, which is less than its effect on HMBA- or Me_2SO-induced differentiation. Thus, although the extent of inhibition varied, TPA inhibited the differentiation of MELC (DS19) induced by three different chemicals: HMBA, Me_2SO, and butyric acid.

We have isolated a clone of MELC (clone 9, derived from DS19) that shows a high incidence (up to 20%) of spontaneous differentiation—that is, differentiation without an inducer added to the culture medium. When TPA was added to cultures of clone 9 MELC, complete inhibition of appearance of benzidine-reactive cells was obtained with concentrations of TPA as low as 2 ng/ml (3 nM). Fifty percent inhibition of spontaneous differentiation was achieved with 0.15 ng/ml (0.25 nM).

The results suggest that spontaneous differentiation of MELC is more sensitive to TPA than is induced differentiation. This may reflect either an inherent difference in the susceptibility of cell lines DS19 and clone 9 to TPA or a difference in the TPA-sensitive step under conditions of spontaneous and induced differentiation.

Table 1. Effect of TPA-related plant diterpenes on cell growth and differentiation of DS19 cells

Compound*	Tumor-promoting activity on mouse skin	Concentrations for 50% inhibition of B^+ cell induction,† ng/ml	Transient growth inhibition‡
TPA	+	12	+
Phorbol	−	>300§	−
PDD	+	3–10	+
4α-PDD	−	>300§	−
Mezereine	NT¶	10–30	+
Ingenol dibenzoate	+	3–10	+

Compounds were added to cultures on day 1, together with 4 mM HMBA, and the extent of differentiation was determined.

* For descriptions of these compounds and their tumor promoting activities, see *text* and refs. 13–17.

† Different concentrations of each of the compounds were added to the culture medium on day 1, together with 4 mM HMBA. The number of benzidine-reactive (B^+) cells was scored on day 4. Inhibition of induction of B^+ cells by 50% is compared to cultures receiving only HMBA. With HMBA alone, 80–90% of the cells were B^+ on day 4.

‡ Transient growth inhibition was measured as described in Fig. 2.

§ Inhibition was not achieved at up to this concentration and was not tested beyond it.

¶ −, Not tested.

Effects of TPA-Related Compounds on Differentiation. TPA belongs to a broad class of plant diterpenes (16). Individual compounds in this class differ considerably in their activity as tumor promoters on mouse skin (15–17) as well as in their ability to alter growth properties of cells in culture (18, 19). To determine whether a correlation exists between tumor-promoting activity and inhibitory effect on differentiation, several compounds were tested for their effects on HMBA-induced differentiation of MELC (DS19) (Table 1). Both TPA and PPD, which are potent tumor promoters (15–17), caused a 50% inhibition of differentiation at a concentration of only 10–12 ng/ml. On the other hand, phorbol itself and 4α-PDD, which are inactive as tumor promoters (15–17, 26), did not inhibit differentiation at concentrations as high as 300 ng/ml. Two other plant diterpenes, mezereine and ingenol dibenzoate, with ring systems different from phorbol, also were potent inhibitors of differentiation. Ingenol compounds are known tumor promoters (16). The compound mezereine, an antileukemic agent isolated from *Daphne mezerum L*, has not to our knowledge, been tested for skin-tumor-promoting activity. It does, however, have other effects in cell culture that resemble those of TPA (19).

Influence of Tumor Promoters on Cell Growth and Reversibility of TPA Effect. Among the several biological properties of the tumor-promoting agents, one commonly observed effect is an initial and transient inhibition of DNA synthesis and cell proliferation (15, 19). TPA caused inhibition of the initial growth rate but not the saturation density of MELC (Fig. 2*A*). HMBA also causes a transient inhibition of DNA synthesis and cell growth (27). Inhibition of cell growth was more pronounced in the presence of both TPA and HMBA than with either agent alone. Growth inhibition was not detected with the inactive tumor-promoting agent, phorbol (Fig. 2*B*). Other tumor promoters and inactive analogs tested showed a

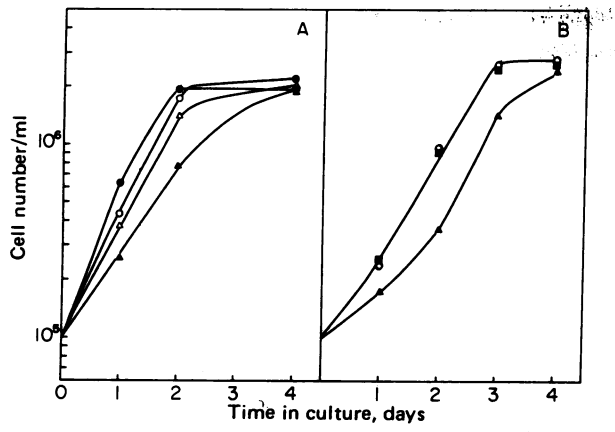

FIG. 2. Effect of HMBA and tumor promoters on growth of DS19 cells. (*A*) DS19 cells were grown as described in Fig. 1 with control medium (●), TPA at 100 ng/ml (△), 4 mM HMBA (○), or TPA at 100 ng/ml plus 4 mM HMBA (▲). The cells were counted on each of the days indicated. (*B*) Cells were grown in 4 mM HMBA alone (▲), 4 mM HMBA plus phorbol at 100 ng/ml (○), or 4 mM HMBA plus TPA at 100 ng/ml (■).

positive correlation among inhibitory effects on cell growth, inhibitory effects on differentiation, and effectiveness as a tumor promoter (Table 1).

To eliminate the possibility that TPA inhibition of HMBA-induced differentiation is simply a nonspecific consequence of growth inhibition, the following experiment was carried out. MELC (DS19) were cultured with TPA (100 ng/ml) for 2 days, washed with fresh medium without TPA, and then grown in fresh medium with or without 4 mM HMBA. The growth and differentiation of these cells were similar to those observed with cells that had not been precultured with TPA. Furthermore, when such TPA-pretreated cells were subsequently cultured with both TPA and HMBA, there was little inhibition of growth rate but a marked inhibition by TPA of the HMBA-mediated induction of benzidine-reactive cells (70–80% inhibition by TPA at 30 ng/ml). These results suggest that the inhibitory effect of TPA on the induction of differentiation by HMBA is not a result of cytotoxic effects on cell growth. These results also indicate that exposure of MELC to TPA for 2 days does not alter their inherent potential to be induced to differentiate by HMBA, once the TPA is washed out. Thus, the inhibitory effect of TPA on differentiation is reversible.

Effect of Time of TPA Addition on HMBA-Inducible Differentiation. An increasing proportion of MELC become committed to differentiate during continuous exposure to inducer (12, 28). Commitment, assayed by scoring the benzidine reaction after transfer of MELC from culture with HMBA to fresh medium without inducer, was detectable within 24 hr of exposure to HMBA and is complete by 48 hr (Fig. 3).

To determine how TPA-mediated inhibition of differentiation is related to commitment, the following experiment was performed. TPA was added to MELC cultures (DS19) at various times after addition of HMBA to the culture. At each of these times, an aliquot of the culture was removed and assayed for commitment to differentiation by transfer to culture medium without inducer. The proportion of benzidine-reactive cells was determined in all cultures after a total of 5 days. The results (Fig. 3) indicate that the addition of TPA could be delayed for up to 24 hr after the addition of HMBA and still maximally inhibit differentiation. Delaying the addition of TPA beyond 24 hr caused a decreasing inhibition of differentiation and this decrease in inhibitory effect was inversely related to the proportion of the cells committed to differentiate. Addition of TPA

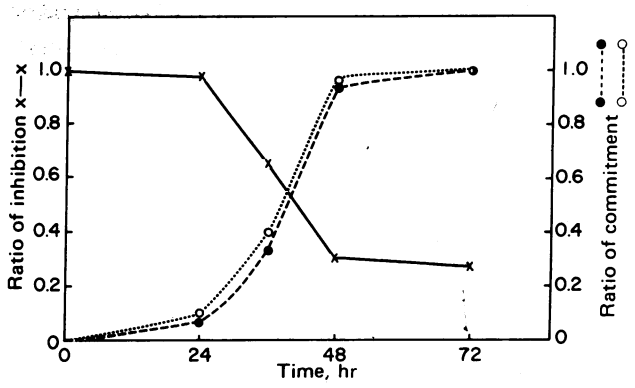

FIG. 3. TPA-mediated inhibition of differentiation and its relationship to commitment. In all cultures, DS19 cells were inoculated into growth medium at 10^5 cells per ml and 4 mM HMBA was added at time 0. To one set of cultures (x), TPA (100 ng/ml) was then added at 0, 24, 36, 48, or 72 hr, and the % B^+ cells was scored on day 5. To other sets of cultures HMBA was added at time 0; aliquots of these cells were transferred, by centrifugation and resuspension, at 24, 36, 48, or 72 hr to fresh medium either free of both HMBA and TPA (○) or free of HMBA but containing PA (100 ng/ml) (●) and the cultures were scored for % B^+ cells on day 5. "Ratio of inhibition" is defined as the % of B^+ cells obtained on day 5 when TPA was added at the indicated time, divided by the % of B^+ cells obtained when TPA was added at time 0. "Ratio of commitment" is defined as the % of B^+ cells obtained on day 5 when HMBA was added at time 0 and removed at the indicated time, divided by the % of B^+ cells obtained when HMBA was added at time 0 and removed at 72 hr.

to the fresh medium to which MELC were transferred, in the assay for commitment, did not affect their capacity to differentiate. This suggests that TPA can inhibit the differentiation of cells that have not yet passed a critical step necessary for commitment. Cells already committed to differentiate are not affected by TPA.

Effect of TPA on Globin mRNA Content. Accumulation of globin mRNA in the cytoplasm precedes the appearance of benzidine-reactive cells in MELC cultures induced to differentiate (7, 29, 30). The effect of TPA on the content of globin mRNA in MELC cells was studied by molecular hybridization using globin [^{3}H]cDNA. To hybridize 50% of globin [^{3}H]cDNA required 0.38 μg of total cytoplasmic RNA from HMBA-treated DS19 cells and 0.93 μg of RNA from the corresponding DS19 cells treated with HMBA plus TPA (Fig. 4*A*). Thus, under these conditions DS19 cells treated with both HMBA and TPA contained about 60% less globin-specific mRNA sequences than DS19 cells treated with HMBA alone. Even in the absence of inducer, there was about 0.5% of spontaneous differentiation; globin mRNA can be found in uninduced DS19 cells at about 1.0% of the level of that found in fully induced cultures (Fig. 4*B* and unpublished data). This level of globin mRNA is also decreased, about 50%, by exposure of DS19 cells to TPA (Fig. 4*B*).

Cells of clone 9 MELC showed a high rate of spontaneous differentiation associated with an approximately 10-fold higher level of globin mRNA than that found in uninduced DS19 cells (Fig. 4*C*). Exposure to TPA decreased the globin mRNA content of clone 9 cells by about 85%.

DISCUSSION

The present results show that TPA and related plant diterpenes known to be tumor promoters on mouse skin inhibit both spontaneous differentiation and differentiation induced by HMBA, Me_2SO, or butyric acid in MELC. Derivatives of these compounds that are inactive in tumor promotion did not inhibit differentiation of MELC. A correlation between the skin-

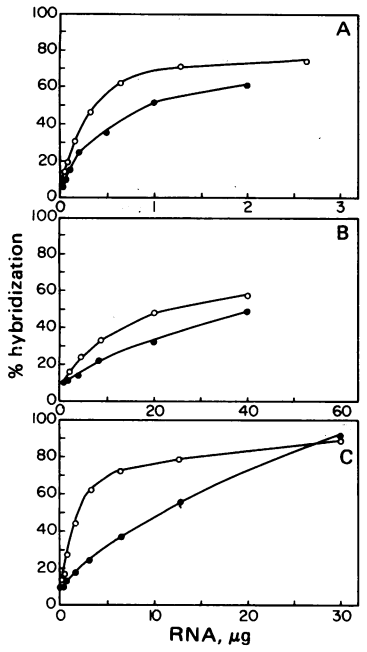

FIG. 4. Accumulation of globin mRNA in HMBA-induced or spontaneously differentiating MELC cultured with (●) or without (○) TPA (100 ng/ml). Total cytoplasmic RNA was extracted from: (*A*) DS19 cells grown in 4 mM HMBA or 4 mM HMBA plus TPA for 3 days as described in Fig. 2; (*B*) DS19 cells grown in control medium or in medium containing TPA for 3 days; (*C*) clone 9 cells grown for 5 days without or with TPA as described in Fig. 1. RNA was assayed for globin mRNA content by hybridization to [^{3}H]cDNA as described in *Materials and Methods*.

tumor-promoting activity of these compounds and their capacity to induce plasminogen activator production in fibroblast cultures has been reported (18, 19). In preliminary studies, we found that plasminogen activator production is also induced when MELC cells are exposed to TPA (unpublished data).

The tumor promoters exert their differentiation-inhibiting effect at or just prior to commitment. In this system, commitment is defined as the ability of an inducible strain of MELC to continue to differentiate even after the inducer has been removed from the culture (12). Delaying the addition of TPA until after committed cells can be detected produces a decreasing inhibitory effect, and the rate of decrease in inhibition is inversely related to the rate of appearance of committed cells. Addition of TPA to cultures after the culture is fully committed does not suppress expression of the erythropoietic program of development. It has been previously shown that differentiation, induced by HMBA, Me_2SO, or butyric acid, is associated with specific metabolic events occuring 4–20 hr after addition of the inducer, and these events may be prerequisites for commitment. They include uptake of inducer (31, 32), prolongation of G_1 and a transient inhibition of DNA synthesis (27), and alterations in membrane properties (33, 34). Apparently, phorbol esters do not act by inhibiting these early events because the addition of TPA can be delayed to 24 hr after addition of the inducer and still inhibit differentiation. The mechanism by which TPA inhibits differentiation is not known. Inhibition of differentiation of MELC by TPA is associated with a decrease of globin mRNA. This may reflect either a block in transcription or processing of globin mRNA or an increase in globin mRNA turnover.

During the course of our studies we learned that Rovera *et al.* (35) also found that tumor promoters inhibit spontaneous differentiation of MELC. Our present work establishes that tumor promoters inhibit both spontaneous and inducible cell differentiation. An inhibitory effect of TPA on differentiation may not be unique to MELC. A recent report indicates that TPA causes a reversible inhibition of myogenesis in chick myoblast cultures (36). Preliminary evidence indicates that differentiation of neuroblastoma cells, induced by serum depletion, dibutyryl adenosine 3′:5′-cyclic monophosphate or BrdUrd, is also inhibited by TPA (D. N. Ishii, E. Fibach, H. Yamasaki, and I. B. Weinstein, unpublished data). It has been recently reported that HMBA (37) and Me_2SO (38) can also serve as inducers for neuroblastoma differentiation. These findings are of particular importance for two reasons. First, a possible mechanism for tumor promotion can be postulated—that is, TPA may act to block the steps involved in terminal differentiation and thus allow unrestrained cell proliferation. Second, the facts that HMBA and Me_2SO can induce several different types of cells to differentiate and that TPA can inhibit differentiation in these diverse cell types suggest that there may be common mechanisms which trigger commitment of cells programmed for a variety of patterns of cellular differentiation.

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