Effect of Tumor Promoter 12-O-Tetradecanoylphorbol-13-acetate on Recovery of Methotrexate-, N-(Phosphonacetyl)-L-aspartate-, and Cadmium-resistant Colony-forming Mouse and Hamster Cells

Ferenc Bojan, Anne R. Kinsella, and Margaret Fox

ABSTRACT

The effect of the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and its nontumor promoting derivative 4-O-methyl-12-O-tetradecanoylphorbol-13-acetate on the frequency of mouse and hamster cells resistant to methotrexate (MTX), N-(phosphonacetyl)-L-aspartate, and cadmium has been examined. TPA alone at concentrations up to 1.0 μg/ml had no significant effect on the plating efficiency of either mouse or hamster cells. Exposure of 3T3 and 3T6 mouse and V79 and Chinese hamster ovary cells at low density to the 3 compounds in the presence of TPA (0.1 μg/ml) did not result in any increase in the recovery of resistant colonies. When plated at high density, exposure to drug selection in the presence of TPA resulted in a 3- to 10-fold increase overall in the incidence of MTX-, N-(phosphonacetyl)-L-aspartate-, and cadmium-resistant mouse cells. However, an increase greater than 3-fold was not observed in hamster cells exposed to drug plus TPA under the same conditions. 4-O-Methyl-12-O-tetradecanoylphorbol-13-acetate had no significant effect on the frequency of MTX-resistant cells. Seventy V79 cell clones surviving MTX (200 to 400 μM) alone and 79 surviving MTX plus TPA were isolated and retested for resistance to MTX. None were stable. In contrast, 6 out of 42 mouse colonies isolated from MTX alone and 55 out of 99 isolated from MTX plus TPA showed stable resistance on retesting in MTX. The implications of these results in relation to possible mechanisms of tumor promotion are discussed.

INTRODUCTION

Resistance of cultured cells in vitro to the specific DHFR inhibitor MTX has been shown to be, in most cases, due to an increase in the number of DHFR gene copies per cell resulting in an overproduction of the DHFR enzyme (1, 12, 15). The extra DHFR genes were found either in small acentric (double minute) chromosomes (10) or integrated within specific homogeneously staining regions of certain chromosomes (3, 4).

Recently, it has been found that the well-known tumor promoter TPA could dramatically increase the incidence of the MTX-resistant, colony-forming mouse cells through the amplification of the DHFR gene (19). Varshavsky (19, 20) has proposed that the TPA alone or together with a cytotoxic drug acts as a "locus-unspecific hrone" which can increase, through extra "illegitimate" rounds of DNA replication, the frequency of amplification of not only the DHFR gene but also of any genes, including the transforming gene(s). Thus, an increase in the dosage of the transforming gene(s) may lead to phenotypic changes, among them malignant transformation (13, 20). This hypothesis of tumor promotion results in several further predictions. (a) If the TPA has a locus-unspecific effect on the gene multiplication, then TPA should increase the frequency of drug-resistant clones in all cases where the resistance, at least in part, is the result of overproduction of the target protein due to gene amplification. (b) Since the in vivo skin tumor-promoting effect of TPA is species specific, the effect of TPA on the gene amplification should differ in cultured cells derived from different species. (c) Malignant transformation is known to be an irreversible process; therefore, the gene amplification should be a stable phenomenon, at least in a proportion of colonies.

In order to test these predictions, the following comparisons were made. (a) We investigated whether or not TPA could enhance the recovery of not only MTX-resistant but also PALA- and cadmium-resistant colony-forming cells. PALA is a transition state inhibitor of aspartate transcarbamylase (11). In PALA-resistant cells, the overproduction of the multifunctional CAD protein, with carbamyl-P synthetase, aspartate transcarbamylase, and dihydroorotase enzyme activities, has been shown to be due to amplification of the CAD gene (21). Cadmium, on the other hand, is detoxified by binding to metallothioneins which are small, cysteine-rich intracellular proteins synthesized by nearly all cultured cells in response to exposure to heavy metals (7, 14). In cadmium-resistant mouse cells, amplification of the MT-I gene has been demonstrated (2). (b) The effect of TPA on the recovery of MTX-, PALA-, and cadmium-resistant colony-forming cells was compared in 2 mouse and 2 hamster cell lines. This was because, although TPA is a potent tumor promoter in mouse skin, it has not been demonstrated to be a promoter on hamster skin (6, 17). (c) The influence of the nonpromoting derivative of TPA, 4-O-Me-TPA on drug resistance was compared with that of TPA. (d) Finally, the stabilities of the MTX-resistant mouse and hamster cells selected in a single step in the presence or absence of TPA, were compared.

MATERIALS AND METHODS

Cells. Uncloned V79 (5) and CHO (8) Chinese hamster cells and 3T6.
(18) and 3T3 (18) mouse cells were used. The cells were cultured in
DMEM (Flow Laboratories Ltd., Irvine, United Kingdom) supplemented
with penicillin (100 units/ml), streptomycin (50 μg/ml), and 10% fetal
bovine serum for hamster cells or 10% calf serum for mouse cells. The
cells were grown in a humidified incubator at 37° with 5% CO2 in air,
gas phase.

Chemicals. TPA and 4-O-Me-TPA (Sigma Chemical Co., St. Louis,
Mo.) were dissolved in dimethyl sulfoxide. Stock MTX (Lederle, Gosport,
United Kingdom) and PALA (NSC 224131) solutions were made up
in phosphate-buffered saline, pH 7.2 (0.25 g KH2PO4·2.16 g Na2HPO4·7
H2O-0.2 g KCl-8.0 g NaCl-0.1 g CaCl2-0.1 g MgCl2·6 H2O). CdSO4
(Hopkin and Williams, Ltd., Chadwell Heath, United Kingdom) was dis-
solved in distilled water. Thymidine and hypoxanthine were obtained
from Sigma Chemical Co. F-MTX was kindly supplied by Dr. A. McGown
of these laboratories.

 Colony Formation Assay. All experiments started before the cells
reached confluence; V79, CHO, and 3T3 were used 48 hr after and 3T6
cells 96 hr after the last subculture. The dishes (9 cm in diameter) were
seeded with 5 x 104 or 5 x 105 cells in DMEM supplemented with 1% ser-
um, and the cells were allowed to attach for 4 hr. After attachment,
this medium was replaced by medium containing 10% serum and the
appropriate test chemicals. The plates were incubated, with no further
change of medium for 7 days (at density of 5 x 105 cells/plate) or for 10
to 20 days (at density of 5 x 104 cells/plate). The colonies were stained
with crystal violet after fixation with 10% formaldehyde in 0.9% NaCl
solution. Two sets of experiments were always performed with 5 plates/
point. All data plotted in Charts 1 to 5 represent the mean results of 2
experiments.

In the experiments in which the cells were seeded at the density of 5
x 105/plate, the ratio of the number of colonies formed in the experimen-
tal plates to that in untreated control plates was calculated as the
surviving fraction. In the experiments at high density (5 x 106 cells/dish),
the incidence of resistant cells, defined as the ratio of the number of
colonies to the number of cells initially seeded, was calculated.

Isolation of Colonies. Colonies were isolated using silicone-greased
plastic isolation rings. The isolated colonies were grown in 6-cm dishes.
The clones selected in the presence of TPA were always grown in
medium containing TPA. After isolation, the putative MTX-resistant
hamster and mouse cell colonies were allowed to grow in MTX-free medium
for 1 and 2 weeks, respectively, before retesting for phenotypic stability
as follows. The clones were subcultured at 1 to 5 x 104 cells/6-cm plate
and exposed to a second MTX challenge at the same concentration as
that in which they were selected. The hamster cells were observed for 2
weeks, and the mouse cells were observed for 3 weeks following this
challenge. Surviving and evenly growing clones were considered to have
stable resistance to MTX.

Quantitation of DHFR Levels. Quantitation of the DHFR levels in
MTX-resistant and control mouse 3T6 cell clones was carried out by
saturation of DHFR with F-MTX, and analysis of the degree of fluores-
cence emission from individual cells was according to the method of
Kaufman et al. (9).

MTX-resistant clones and control clones were subcultured into 25-sq
cm flasks and allowed to grow in MTX-free medium for at least 6 days.
Prior to the attainment of confluence, the cells were fed with 2 ml of
DMEM containing 3 x 10-4 M F-MTX, thymidine, and hypoxanthine
and incubated for 22 hr. The cells were then rinsed and incubated in 5 ml
DMEM for 2 hr to allow for the efflux of unbound F-MTX. The cells were
gently trypsinized, suspended in 5 ml DMEM, and centrifuged at 800
rpm for 5 min. The pellet was gently resuspended in cold phosphate-
buffered saline, the cell number was adjusted to 1 x 108 cells/ml, and
the samples were maintained at 4° until they were analyzed for fluores-
cence. A FACS (FACS IV; laser power, 220 milliwatts; photomultiplier
tube, 700 V; Becton Dickinson and Co.) was used to analyze cells for
fluorescence. For each determination, at least 30,000 cells were run
through the FACS at a flow rate of approximately 500 cells/sec. The
DHFR was expressed as the channel number of the peak of fluorescence.

RESULTS

Effect of TPA Alone on Plating Efficiency. TPA by itself, at
concentrations up to 1.0 μg/ml, did not influence the plating
efficiency of cell lines significantly (data not shown).

Effect of TPA on the Recovery of Resistant Colonies at
Low Cell Densities. In this series of experiments, we investigat-
ed the effect of TPA on the relative plating efficiency of V79,
CHO, 3T6, and 3T3 cells plated at the density of 5 x 105 cells/plate
and treated with MTX, PALA, or cadmium. The results
summarized in Charts 1 to 3 show that the surviving fraction of
the hamster and mouse cells exposed to different concentrations
of MTX, PALA, and cadmium did not change significantly in the
presence of TPA. It is to be noted that the sensitivity of the
different cell lines to MTX and PALA did not differ greatly;
however, considerable differences could be observed in the
sensitivities of cell lines to cadmium (Chart 3).

Effect of TPA on the Recovery of Resistant Colonies at
High Cell Densities. In this series of experiments, the effect
of TPA on the recovery of MTX-, PALA-, and cadmium-resistant
clones of hamster and mouse cells at the density of 5 x 106 cells/plate was studied. TPA increased the incidence of MTX-
resistant hamster cells not more than 3-fold (Chart 1). The overall
recovery of the MTX-resistant mouse cells (at 100 to 300 nM
MTX for 3T6 cells, 200 to 400 nM MTX for 3T3 cells) was,
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Surviving fraction of MTX-resistant Clones. Finally we studied the stability of MTX resistance in isolated V79 and 3T6 clones. The results, summarized in Table 1, show that none of the hamster cell lines selected at different concentrations of MTX with or without MTX.

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TPA also did not increase the frequency of the PALA-resistant hamster cells to any significant extent, whereas it enhanced the incidence of PALA-resistant mouse cells by 3- to 4-fold (Chart 2).

The overall effect of TPA on the incidence of cadmium-resistant colony-forming cells was also species dependent (Chart 3). TPA increased the recovery of the cadmium-resistant cells to a greater extent in mouse than in hamster cells, although TPA also had a notable effect on V79 cells at the lowest cadmium concentration. The considerable differences in the sensitivity of the different cell lines to cadmium toxicity were also evident in this experiment (Chart 3).

The effect of TPA was dose dependent and specific. TPA (0.01 μg/ml) had only a negligible effect on the incidence of MTX- and cadmium-resistant V79 and 3T6 cells, while the effect of a 1.0-μg/ml dose of TPA hardly exceeded that of a 0.1-μg/ml dose of TPA which was used in the previous experiments (Charts 4 and 5). The nonpromoting analogue 4-O-Me-TPA at a concentration of 0.1 μg/ml did not influence the incidence of the drug-resistant colony-forming cells (Charts 4 and 5). In one experiment, 3T6 cells were pregrown in medium containing TPA for 96 hr before reseeding and exposure to MTX and TPA (Chart 4). In this case, the effect of TPA on the incidence of resistant cells was slightly greater than in cells exposed to TPA simultaneously with MTX.

Chart 2. Effect of TPA on recovery of PALA-resistant colony-forming mouse and hamster cells. O, without TPA; •, with TPA (0.1 μg/ml).

However, 3- to 10-fold higher in the presence of TPA (Chart 1). TPA also did not increase the frequency of the PALA-resistant hamster cells to any significant extent, whereas it enhanced the incidence of PALA-resistant mouse cells by 3- to 4-fold (Chart 2).

The overall effect of TPA on the incidence of cadmium-resistant colony-forming cells was also species dependent (Chart 3). TPA increased the recovery of the cadmium-resistant cells to a greater extent in mouse than in hamster cells, although TPA also had a notable effect on V79 cells at the lowest cadmium concentration. The considerable differences in the sensitivity of the different cell lines to cadmium toxicity were also evident in this experiment (Chart 3).

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Chart 3. Effect of TPA on recovery of cadmium-resistant colony-forming mouse and hamster cells. O, without TPA; •, with TPA (0.1 μg/ml).

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Chart 4. Recovery of MTX-resistant colony-forming mouse and hamster cells in presence of different doses of TPA and effect of 4-O-Me-TPA. O, MTX only; □, 0.1% dimethyl sulfoxide; ○, 4-O-Me-TPA (0.1 μg/ml); □, TPA (0.01 μg/ml); •, TPA (0.1 μg/ml); △, TPA (1.0 μg/ml); ⬤, pregrown with TPA (0.1 μg/ml) for 96 hr before being exposed to MTX plus TPA (0.1 μg/ml). Density, 5 x 10^5.
without TPA could survive the second MTX treatment after 1 week of growth in MTX-free medium. It is to be noted that we did not consider clones to be resistant which, by the end of the 2 weeks of observation, formed numbers of colonies similar to those in experiments in which the original selection of resistant cells was carried out.

Fourteen % of mouse clones selected without TPA and 55% of the clones selected in the presence of TPA survived the second MTX-exposure and grew evenly in the plates after a 2-week period in MTX-free medium (Table 1).

Quantitation of the DHFR Levels in Stably Resistant Clones. FACS analysis of F-MTX-labeled MTX-resistant and control 3T6 cell clones demonstrated quite convincingly that MTX resistance was not conferred on either selection group (150 nm MTX plus TPA (0.1 μg/ml) or 150 nm MTX minus TPA) by gene amplification (Chart 6). The DHFR level was considered to be changed if the peak of fluorescence was outside the mean ±2 S.D. of the 4 control cell populations. Only 3 of the 20 clones isolated in the presence of MTX plus TPA showed increased DHFR levels (Chart 6) compared with 8 of 18 clones isolated in MTX alone. In fact, overall, the clones selected in the presence of TPA showed a greater proportion with reduced DHFR levels than those selected in MTX alone.

DISCUSSION

Varshavsky (19) observed that the potent tumor promoter TPA increased the incidence of MTX-resistant colony-forming 3T6 cells by up to 100-fold. In our experiments at high density (5 x 10^5 cells/plate), TPA also enhanced the recovery of MTX-resistant 3T6 cells, but its effect was not so dramatic. We found no more than a 15-fold increase in the incidence of MTX-resistant 3T6 cells in the presence of TPA (Chart 4). This considerable difference in results obtained may be due to different experimental procedures and may indicate that the yield of MTX-resistant cells selected in the presence of TPA is strongly influenced by such variations in technique.

We allowed the cells to attach before treatment and used the cells before they reached confluency. In contrast, Varshavsky (19) harvested the cells from confluent cultures and then plated the cells into medium containing MTX and TPA; the influence of both drugs on cell attachment is, however, unknown.

We found that TPA increased the incidence of not only MTX-resistant but also PALA- and cadmium-resistant 3T3 and 3T6 cells, and this effect was in all cases density dependent. We suggest that this may be due to release of some metabolites by dying cells which can rescue a number of cells at higher cell density until the survivors develop some mechanism of resistance and this process is promoted by TPA. In this postulated rescue of cells at high density, the role of metabolic cooperation may be excluded because TPA is known to inhibit the metabolic cooperation between cells cultured in vitro (22).

TPA treatment had considerably greater overall effects on the recovery of drug-resistant cells in mouse cells compared with hamster cells. This is in good agreement with the in vivo observations; TPA is a potent tumor promoter in mouse skin but it is ineffective as a promoter in hamster skin (6, 17). Moreover, Shoyab et al. (16) failed to detect any notable activity of phorbol-12,13-diester 12-ester hydrolase in mouse skin. This enzyme, however, shows high activity in hamster skin and is thought to convert the biologically active phorbol-12,13-diesters, among

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**Chart 5.** Recovery of cadmium-resistant colony-forming mouse and hamster cells in the presence of different doses of TPA and effect of 4-O-Me-TPA. O, MTX only; Q, 0.1% dimethyl sulfoxide; 3, 4-O-Me-TPA (0.1 μg/ml); □, TPA (0.01 μg/ml); ◇, TPA (0.1 μg/ml); Δ, TPA (1.0 μg/ml). Density, 5 x 10^5.

**Table 1**

<table>
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<tr>
<th>Conditions of selection</th>
<th>No. of isolated clones</th>
<th>No. of surviving clones</th>
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<td><strong>V79S clones</strong></td>
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**Chart 6.** DHFR levels in MTX-resistant and control (contr.) mouse 3T6 clones. Clones resistant to 150 nm MTX were selected in the absence of TPA (C) or in the presence of TPA (0.1 μg/ml) ( ◇); 4 control cell populations; bars, S.D.
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them TPA, to the inactive phorbol-13-monoester. According to the in vivo results, one can suppose that the in vitro cultured hamster cells retain their ability to inactivate the TPA, whereas mouse cells are not able to produce phorbol-12,13-diester hydrolyase to detoxify the TPA.

The MTX resistance of hamster cells was not stable, irrespective of the presence of TPA during the selection. In the absence of the selective pressure of MTX, all the 149 clones lost their resistance within approximately 15 cell cycles. However, a considerable number of the 141 mouse clones retained their MTX resistance without selective pressure for 15 cell cycles. There was a much higher number of stable MTX-resistant mouse cell lines when selection was carried out in the presence of TPA, which shows that the TPA can facilitate not only the development but also the stabilization of the drug-resistant phenotype.

Overproduction of target protein has been found to operate through gene amplification in the development of resistance to all 3 chemicals tested in our experiments (2, 12, 15, 19, 21). If the drug resistance in our experiments was also due to gene amplification, it may be supposed that the TPA alone or together with cytotoxic drugs can facilitate the mouse cells to amplify their genes randomly, including their transforming gene(s), and to preserve these amplified genes results in a stable phenotype. However, before such deductions can be made with complete confidence, more information on the exact nature of the drug-resistant phenotype is necessary. Indeed, preliminary FACS analysis of the isolated clones after exposure to fluorescein-labeled MTX (Chart 6) indicated that very few of the mouse colonies exhibit stable resistance to MTX due to increased production of the DHFR enzyme and therefore gene amplification.

In conclusion, therefore, we have tested the hypothesis that the potent tumor promoter TPA acts as a "locus-unspecific firone," which can randomly increase the frequency of amplification of genes, including the transforming gene(s). TPA considerably increased the recovery of MTX-, PALA-, and cadmium-resistant clones of 3T3 and 3T6 mouse cells but had little effect in V79 and CHO hamster cells. The effect of TPA was cell density and dose dependent, and the nonpromoter 4-O-Me-TPA had no TPA-like effect. The MTX-resistant V79 hamster cell colonies selected with or without TPA lost their resistance within 15 cell cycles; however, a large number of 3T6 mouse cell clones, 55% of all those selected and maintained in the presence of TPA, preserved their resistance for more than 15 cell cycles. At this point in time, the authors recommend caution in interpreting the influence of TPA on drug resistance as being due to its role as a locus-unspecific firone.

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REFERENCES

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