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Differential Effects of 12-*O*-Tetradecanoylphorbol-13-acetate on Cultured Normal and Neoplastic Human Bronchial Epithelial Cells

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ABSTRACT

The effects of the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on 10 human lung carcinoma cell lines were compared to those seen on normal human bronchial epithelial (NHBE) cells. TPA (0.1 to 100 nM) did not enhance the clonal growth rate for any of the cell lines. As little as 3 nM TPA induced the NHBE cells to undergo terminal squamous differentiation and thus completely inhibited their proliferation; in contrast, none of the carcinoma cell lines was significantly inhibited at this concentration, and they all continued to proliferate in as much as 100 nM TPA. To determine if this lack of TPA inhibition of clonal growth reflected resistance to TPA induction of terminal squamous differentiation, we measured the ability of TPA to induce cross-linked envelope formation and to increase plasminogen activator activity in four carcinoma cell lines. Cross-linked envelopes were not induced in two lines, and only a small number were induced in the other two lines relative to NHBE cells; plasminogen activator activity was induced in NHBE cells but not in any of the cell lines.

INTRODUCTION

In the multistage model of carcinogenesis, cells that have been initiated through exposure to a nontumorigenic dose of a carcinogen will become malignant if subsequently exposed to repeated doses of a tumor promoter (3). TPA,[2] a phorbol ester found in croton oil, was the first tumor promoter isolated (12). Through extensive research using experimental animals and their cultured cells, it is known that TPA has pleiotropic effects (1, 2, 5, 27, 30), but it is not clear which of these effects is necessary and/or sufficient for promoter activity. Furthermore, due to wide interspecies and even interstrain differences in susceptibility to tumor promotion in experimental animals (4, 24), both qualitative and quantitative extrapolation of these results to human systems would be uncertain (10). In a previous report (28), we described some of the effects of TPA and the indole alkaloid tumor promoter, teleocidin B, on NHBE cells. The effects were squamous morphology change, induction of CLE formation, increased secretion of PA, cessation of cell division, and inhibition of ornithine decarboxylase activity. Thus, we concluded that TPA and teleocidin B induce terminal squamous differentiation of NHBE cells.

In a recently proposed model of carcinogenesis, initiated cells are resistant to TPA-induced terminal differentiation and therefore have selective growth advantage over surrounding normal cells (13, 31). Consistent with this model, TPA induces rapid terminal squamous differentiation in a subpopulation of cultured normal murine epidermal keratinocytes (7, 11, 21, 32) and the entire population of human keratinocytes (21). Furthermore, malignant human keratinocyte cell lines are less sensitive to TPA-induced differentiation (22). Thus, it was of interest to examine the effects of TPA on differentiation in several human lung carcinoma cell lines. We speculated that lung carcinoma cells may be resistant to TPA-induced differentiation and that this property could serve as a useful selective condition and marker of transformation in *in vitro* carcinogenesis experiments.

MATERIALS AND METHODS

Chemicals and Reagents. Teleocidin B was a gift from Dr. T. Sugimura and Dr. H. Fujiki, National Cancer Center Research Institute, Tokyo, Japan. We purchased TPA from Chemicals for Cancer Research, Inc., Eden Prairie, MN; epidermal growth factor and human fibronectin, from Collaborative Research, Waltham, MA; 6- and 24-well plastic culture plates from Costar, Cambridge, MA; Lux 60-mm plastic culture dishes from Miles Laboratories, Inc., Naperville, IL; RPMI 1640, LHC basal medium (18) and FBS from Biofluids, Rockville, MD; trypsin from Worthington Diagnostics Inc., Freehold, NJ; and Vitrogen from The Collagen Corp., Palo Alto, Ca.

Cell Culture Methods and Cell Lines. Normal human bronchial tissue was obtained from donors at the time of autopsy, and NHBE cell outgrowths were cultured as described previously in detail (15, 16). The lung carcinoma cell lines used and their sources were: A-549, A-427, Calu-6, Calu-1, and SW-900 (American Type Culture Collection, Rockville, MD); SK Lu-1 (J. Fogh, Sloan-Kettering Institute for Cancer Research, Rye, NY); NCI H292 (A. Gazdar, NCI-Navy Medical Oncology Branch, Bethesda, MD); and A-2182 and A-1146 (S. Aaronson, National Cancer Institute, Bethesda, MD). They were routinely passaged in either RPMI 1640 with 10% fetal bovine serum (Calu-6, SK Lu-1, NCI H292, Calu-1, A-1146) or GDS medium (25) (A-427, A-1188, A-549, A-2182, and SW-900).

Clonal Growth Assays. NHBE cells are induced to differentiate in LHC-8 medium containing more than 2% serum, whereas the carcinoma cell lines used in this study grow poorly in serum-free LHC-8 media (17). All of these lines grow maximally in media containing 8% FBS (17) and grow well enough for clonal growth assay in 1% FBS. Therefore, all clonal growth assays were conducted in LHC-8 (18) medium containing 1% serum. NHBE cells and carcinoma cell lines were inoculated into coated (fibronectin, 10 μg/ml; Vitrogen, 30 μg/ml; BSA, 10 μg/ml) 60-mm plastic culture dishes (15) at clonal density (2000 cells/dish for the A 549 cell line and 5000 cells/dish for NHBE cells and all the other cell lines). After 24 hr of incubation, cells were exposed to various concentrations of TPA. The media were replaced after 4 days, and after 7 days the plates were washed with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered salt solution (16), the cells were fixed with 10% formalin and stained with 1% crystal violet. The average number of cells per colony was determined with the aid of a computerized image analyzer (Artec 800). The clonal growth rate (population doublings per day) was determined by computing the $\log_2$ of the average number of cells per colony and dividing by the number of days in culture (15). For each assay, 18 random colonies were counted from 2 replicate dishes. Student's *t* test was applied to determine the significant differences between experimental data.

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[2] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; NHBE, normal human bronchial epithelial cells; CLE, cross-linked envelopes; PA, plasminogen activator; FBS, fetal bovine serum.

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Plasminogen Activator Assay. Cell-mediated conversion of plasminogen to plasmin was determined by measuring the plasmin-catalyzed release of [^{14}C]anilide from benzyloxycarbonylglycylprolylarginyl[^{14}C]anilide as described previously (28). Cells were washed 3 times with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered salt solution buffer to remove serum, which contains plasmin, and exposed to TPA in serum-free medium.

CLE Assay. Assays for the presence of CLEs in cell populations were conducted using a modification of method of Sun and Green (9, 26), as described previously (28).

RESULTS

Effects of TPA on Clonal Growth Rates. TPA dose-response experiments (Chart 1) revealed that 3 nM TPA completely inhibited NHBE cell clonal growth in medium containing 1% serum; we reported similar results using serum-free medium (28). In contrast, TPA caused ony a small decrease in clonal growth in the 10 carcinoma cell lines, and all 10 lines can be incubated in medium containing 100 nM TPA for at least 5 subculturings. TPA did not enhance the clonal growth rate for any of the lines at the concentrations tested, and at 1 μM was cytotoxic. The effect of teleocidin B (0.1 to 100 nM) on the clonal growth rate of 3 carcinoma cell lines (A-549, A-1188, and Calu-6) was also assessed; even at a concentration of 100 nM, it inhibited clonal growth by only 30%.

Effects on PA Activity and CLE Formation. NHBE cells secrete PA at a higher constitutive level than the cancer cell lines tested: A-549; Calu-1; NCI H292; and SW-900 (Table 1). After exposure to TPA (100 nM) for 6 hr, the level of PA activity associated with NHBE cells increases from 2.4 ± 1.3 (S.D.) to 5.6 ± 1.2 nmol [^{14}C]anilide released per mg protein per hr. In contrast, TPA has little effect on the PA activity of the carcinoma cell lines.

Neither the NHBE cells nor any of the carcinoma cell lines tested forms a substantial number of CLEs when incubated in LHC-8 medium with 1% serum (Table 1). After exposure to TPA (100 nM), CLE formation in the NHBE cell population increases from 0.4 ± 0.2% to 16 ± 1.5%. In contrast, there is no increase in the formation of CLE in 2 of the cancer cell lines tested (SW-900 and Calu-1) and only a small increase in A-549 and NCI H292, from 0.3 ± 0.2% to 4.0 ± 0.1% and 0.3 ± 0.2% to 4.0 ± 0.1%, respectively.

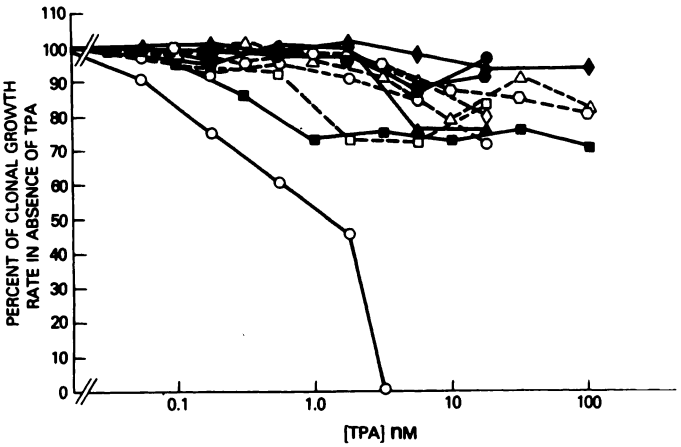

Chart 1. TPA dose-response studies in LHC-8 + 1% serum. For each cell line, cells were inoculated at clonal density. After 24 hr, the cells were exposed to varying concentrations of TPA in 0.1% dimethyl sulfoxide. The medium was changed after 4 days of incubation, and after 7 days cells were fixed with 10% formalin and stained with 1% crystal violet. Number of cells per colony was counted using an Artec 800 computerized image analyzer. Clonal growth rates were determined as described in "Materials and Methods." Control clonal growth rates for NHBE cells and each of the cancer cell lines are in parentheses: A-1188 (0.88), ●; A-549 (1.05), ◆; A-427 (0.35), ●; SK Lu-1 (0.41), ▲; Calu-1 (0.69), ■; NCI H292 (0.70), ○; A-1146 (0.45), ◇; A-2182 (0.91), O– – –O; SW 900 (0.76), △; Calu-6 (0.71), □; NHBE cells (0.95), O——O.

Table 1
Effects of TPA on NHBE cells and carcinoma cell lines

Cell type	Tumor histology	TPA (100 nM)[a]	PA[b]	CLE[c]
NHBE		–	2.4 ± 1.3[d]	0.4 ± 0.2
		+	5.6 ± 1.2[e]	16.0 ± 1.5[e]
A-549	Adeno	–	0.8 ± 0.2	0.3 ± 0.2
		+	0.9 ± 0.3	4.0 ± 0.1[e]
Calu-1	Squamous	–	0.7 ± 0.4	0.3 ± 0.2
		+	0.8 ± 0.3	0.4 ± 0.2
NCI H292	Mucoepidermoid	–	0.9 ± 0.4	0.3 ± 0.2
		+	0.9 ± 0.2	4.0 ± 0.1[e]
SW-900	Squamous	–	0.7 ± 0.3	0.2 ± 0.1
		+	0.7 ± 0.1	0.4 ± 0.2

[a] Media were replaced 6 hr prior to assay.
[b] PA, nmol [^{14}C]anilide released/mg protein/hr; –, no TPA in medium; +, TPA in medium.
[c] Percentage of cell population producing CLE.
[d] Mean ± S.D.
[e] Significant differences ($p < 0.05$) by Student's t test. All experiments were done at least twice.

DISCUSSION

The phorbol ester tumor promoters have varied effects on differentiation of cells in culture; in some systems, TPA inhibits differentiation while in others it has an inducing effect (30). In mouse keratinocyte systems, TPA is mitogenic for one subpopulation of cells but induces terminal squamous differentiation in another subpopulation (11, 21, 32). These findings led to the development of a model of carcinogenesis in which tumor promoters induce terminal differentiation in normal cells, whereas initiated cells are resistant to this effect (13, 31). If this model is correct, one would predict that lines derived from carcinomas are resistant to inducers of terminal differentiation. Indeed, it has recently been reported that human epidermal squamous carcinoma cell lines do not terminally differentiate as quickly as normal epithelial cells when suspended in methocel (22) and that bronchial carcinoma cell lines are resistant to induction of differentiation compared to NHBE cells when exposed to serum (17).

We report here that in the presence of TPA (100 nM), human lung carcinoma cell lines continue to proliferate, few or no CLEs are induced, only small reversible morphological changes are induced (data not shown), and PA activity is not increased. Based on these results we conclude that, in contrast to NHBE cells, human lung carcinoma cell lines are resistant to TPA-induced terminal differentiation. In these experiments, there was no correlation between the histological type of the primary cancer and the response of the derived cell line to TPA. Since we obtain similar results with teleocidin B, a tumor promoter with a completely different structure from TPA, these differences are not due simply to different rates of TPA metabolism.

Increased expression of PA activity has been correlated with the tumorigenicity of fibroblasts transformed with viruses, with

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tumorigenic potential of malignant melanoma cells; and with tumor promoter treatment of normal and tumor virus-transformed cell cultures (6, 8, 23). Most information on the relationship between PA secretion and cancer has come from studies on mesenchymal tissues. There is now accumulating evidence that PA is secreted physiologically in human keratinocytes, and that this secretion increases as the cells approach terminal differentiation (14). Recently, investigations of the correlation of PA secretion with cancer have been conducted in several human epithelial tissues including those of the lung (19, 20), and it has been determined that human lung tumors secrete more PA than adjacent normal lung. These results may have less to do with the relative malignant nature of the tissues studied than with the fact that the majority of a lung tumor is composed of epithelial cells, while normal bronchial tissue contains only one layer of epithelial cells, and is primarily composed of mesenchymal cells and stroma. Because normal human mesenchymal tissues secrete much less PA than do normal epithelial cells (29),[3] a comparison between homogenized primary human lung tumor and homogenized normal human lung may be misleading.

In conclusion, we have shown that resistance to TPA-induced terminal differentiation is correlated with malignant transformation of bronchial epithelial cells. Although the mechanism of TPA action remains unclear, this compound may be useful for selecting preneoplastic and neoplastic cells during *in vitro* carcinogenesis experiments.

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