Enhanced Induction of the Anchorage-independent Phenotype in Initiated Rat Tracheal Epithelial Cell Cultures by the Tumor Promoter 12-O-Tetradecanoylphorbol-13-acetate

Vemon E. Steele,1 Diane K. Beeman, and Paul Nettesheim

Laboratory of Pulmonary Function and Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

ABSTRACT

The purpose of the studies reported here was to compare the response of noninitiated and initiated primary rat tracheal epithelial (RTE) cell cultures to the mouse skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). The endpoints measured were number of cells per culture, colony-forming efficiency, subculturability, and colony formation in soft agarose. Primary RTE cell cultures were exposed on Day 1 to either 0.2% dimethyl sulfoxide, or to 0.1 µg/ml of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). Thereafter, the same cultures were exposed twice weekly from Days 6 to 30 to either 0.2% dimethyl sulfoxide or to TPA (10 µg/ml). Sequential exposure to MNNG and TPA did not increase the number of viable cells per culture beyond that seen in MNNG-exposed cultures. Determination of the frequency of colony-forming cells 10 days after the end of the initiation-promotion treatment (Day 40 of culture) revealed a marked enhancement in colony-forming efficiency of treated cultures compared to dimethyl sulfoxide-exposed control cultures. However, sequential exposure to MNNG and TPA had an additive or slightly more than additive effect on the colony-forming efficiency of RTE cells exposed to MNNG or TPA only.

Treatment of the primary cultures with MNNG alone or TPA alone increased the subculturability of RTE cells to a similar extent. The sequential exposure to MNNG followed by TPA appeared to have an additive effect on the frequency of subculturability.

The most pronounced effect of the sequential MNNG-TPA exposure as compared to single-agent exposure was a marked enhancement of the anchorage-independent (ag⁺) phenotype. Of the cultures treated with MNNG followed by TPA, over 50% were ag⁺ at 60 days. In contrast, of the cultures treated either with MNNG alone or with TPA alone, only 9% were ag⁺ on Day 60. (All control cultures were ag⁻.) Colony-forming efficiency in soft agarose also increased disproportionately between 60 and 120 days in initiated-promoted cultures.

These experiments indicate that the major effect of the tumor promoter TPA on initiated RTE cell cultures is to enhance the appearance of the late ag⁺-phenotype.

INTRODUCTION

Epidemiological evidence strongly suggests that cancer of the respiratory tract is a multistep process (1, 3, 10). To identify the stages of malignant progression and to understand the driving forces which move cells from one stage to the next, cell culture models using respiratory tract epithelium have been developed (8, 12, 16). In previous studies, an organ culture-cell culture system was used to demonstrate that RTE² could be malignantly transformed in culture by exposure to MNNG (11, 14). We also demonstrated that transformation could be significantly enhanced by subsequent exposure to the tumor promoter TPA (15). This promotion-like enhancement of malignant transformation has also been shown for RTE in vivo (18, 21). We have also established previously that normal tracheal epithelial cells respond to TPA by a dose-dependent increase in DNA synthesis (12) and a permanent alteration in in vitro growth which permits the establishment of nonneoplastic cell lines (13). The ability of promoters to induce cell lines in cultured RTE correlated well with their known in vivo promoting activity (17).

Our laboratory has refined and quantitated the tracheal epithelial cell culture system to study carcinogenesis by exposing known numbers of target cells to carcinogens in a monolayer primary system (8, 20). The study reported here demonstrates that TPA markedly enhances the development of the anchorage-independent phenotype in initiated RTE cell cultures. No other significant differences were observed in the response of uninitiated and initiated RTE cells to the tumor promoter TPA.

MATERIALS AND METHODS

Chemicals. MNNG, protease (type VI), human transferrin, bovine insulin, hydrocortisone, and agaron (type II) were purchased from Sigma Chemical Co., St. Louis, MO. TPA was prepared by Chemicals for Cancer Research, Eden Prarie, MN. Type I collagen (Vitrogen) was obtained from the Collagen Corporation, Palo Alto, CA. Ham’s F-12 and Eagle’s and Dulbecco’s minimal essential medium, calcium- and magnesium-free Hanks’ balanced salt solution, and trypsin-EDTA were obtained from Grand Island Biological Co., Grand Island, NY. Insulin, hydrocortisone, 5 ng of transferrin per ml, and 0.3% bovine hypothalamus extract were prepared by Hormone Research, Eden Praire, MN. Type I collagen (Vitrogen) was obtained from the Collagen Corporation, Palo Alto, CA. Ham’s F-12 and Eagle’s and Dulbecco’s minimal essential medium, calcium- and magnesium-free Hanks’ balanced salt solution, and trypsin-EDTA were obtained from Grand Island Biological Co., Grand Island, NY. Sterile Systems, Logan, UT, supplied the FBS, and Microbiological Associates, Walkersville, MD, supplied the newborn calf serum.

Tracheal Epithelial Cell Culture. Epithelial cells were enzymatically removed from the tracheas of specific-pathogen-free Fischer 344 rats by Pronase and cultured on collagen-coated dishes by methods reported previously by Wu et al. (23). Tracheas were filled with 1% Pronase in minimal essential medium (calcium- and magnesium-free) and incubated overnight at 4°C. The cells were collected with minimal essential medium plus 10% FBS, filtered through a 100-µm-pore Nitex filter, centrifuged, and resuspended in growth medium. To remove cell clumps, the suspension was filtered through a 25-µm-pore Nitex filter, and the cell concentration was determined by hemacytometer counting; 40,000 cells/60-mm dish were plated. The cultures were grown in a modified Ham’s F-12 medium (8), containing equal parts of Ham’s F-12 and 3T3 conditioned Dulbecco’s minimum essential medium with 2% FBS. The growth medium was supplemented with 10 µg of insulin per ml, 10⁻⁸ M hydrocortisone, 5 µg of transferrin per ml, and 0.3% bovine hypothalamus extract.

Received March 13, 1984; accepted July 27, 1984.

[1] To whom requests for reprints should be addressed, at Northrop Services, Inc., Box 12313, Research Triangle Park, NC 27709.

[2] The abbreviations used are: RTE, rat tracheal epithelium; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; TPA, 12-O-tetradecanoylphorbol-13-acetate; FBS, fetal bovine serum; CFE, colony-forming efficiency; DMSO, dimethyl sulfoxide.
extract. The final serum concentration in the medium was 1%, low enough to inhibit fibroblast growth. For cell passages 5 to 10, the ratio of conditioned medium to F12 was reduced from 1:1 to 1:3, thus reducing the serum concentration to 0.5%.

Chemical Exposure. The stock solutions of MNNG and TPA were prepared in DMSO and stored at -20°C in the dark. On Day 1 of culture, the growth medium was removed and replaced with serum-free minimal essential medium containing 0.1 μg of MNNG per ml for 1 hr. The cultures were rinsed twice following exposure with minimal essential medium, and then growth medium was added. On Days 6, 10, 13, 17, 21, 24, and 28, the cultures were exposed to 10 pg of TPA per ml of minimal essential medium for 1 hr. The final DMSO concentration in cell exposure media was 0.2%.

Cell Number and CFE Measurement. At Day 40, all cultures were dissociated by rinsing with calcium-magnesium free Hanks’ balanced salt solution. Then 0.2% trypsin-EDTA was added for 10 min at 37°C. The cells were collected, and an equal volume of 20% newborn calf serum in Hanks’ balanced salt solution was added at 4°C. Then the cells were centrifuged and resuspended in growth medium. An aliquot was taken for a direct cell count by hemacytometer, and cell viability was determined by trypan blue exclusion.

The cells were then plated in 0.15 ml of growth medium directly into uncoated multiwell (96-well) dishes (Falcon Labware, Oxnard, CA). The cells from each culture were plated in duplicate. Seven days after plating, the cells were fixed in methanol and stained with Giemsa stain. Colonies greater than 8 cells were counted under a dissecting microscope.

Subculturability Measurement. Primary cultures were subcultured on Day 40 by transferring 10⁵ viable cells into an uncoated 35-mm dish. After this secondary culture became confluent, all cells were transferred to two 60-mm dishes, and 1:10 splits of the cultures were made at each passage thereafter. All cultures reaching confluence for the fifth time at Day 90 after the start of the experiment were considered “subculturable.”

Anchorage Independence Measurement. At Day 60 (passage 2 or 3), the available cultures were dissociated and assayed for anchorage-independent growth by measuring CFE in soft agarose by a modified MacPherson method (4). We suspended 5 x 10⁴ cells in 1.5 ml of 0.3% agarose in growth medium and layered it over a 0.5% agarose base in growth medium. The agarose cultures were kept from drying by adding 0.5 ml of growth medium weekly. At 3 weeks, colonies were scored by phase microscopy.

RESULTS

Based on previous initiation-promotion experiments using organ cultures (15) and previous experiments using single and multiple carcinogen exposures to primary cell cultures (8), an experimental protocol was designed as shown in Chart 1. This protocol describes the timing of the MNNG and TPA exposures and of the various measurements made during the course of the study.

Effect of TPA on Growth of Normal RTE Cells. Before investigating the effects of TPA on MNNG-initiated primary RTE cell cultures, it was important to obtain information on its effects on the growth of normal uninitiated cells. In one study, freshly isolated cells were exposed continuously to various concentrations of TPA starting at 24 hr after plating 10⁵ cells/35-mm dish, and the number of colonies formed was counted on Day 6 of culture. As can be seen in Chart 2, TPA concentrations between 0.01 and 1.0 μg per ml of TPA had no effect on CFE; however, 2 μg/ml and above were severely toxic. Previous experiments (12, 15) with the tracheal explant-out growth system suggested that stimulatory effects of growth might be best obtained with repeated short-term rather than continuous exposures to TPA. We therefore treated primary RTE cell cultures with repeated 1-

hr pulses of TPA using different TPA concentrations. The number of cells per dish was determined after seven 1-hr exposures to TPA, administered between Days 6 and 30 of culture. As the data summarized in Chart 3 show, significant increases in cell number occurred in 30-day-old cultures treated intermittently with very low concentrations of TPA ranging from 0.1 pg to 1.0 ng per ml. Concentrations of 100 ng/ml appeared to be inhibitory to growth. In a separate experiment, it was determined whether TPA induces subculturability or “cell line status” when used in a similar range of concentrations. As we have discussed earlier (5, 13), plateau-phase RTE cell cultures cannot be readily subcultured repeatedly unless they have been altered in their growth capacity by agents such as TPA or by carcinogens. The data summarized in Table 1 show that repeated treatment of cultures with 0.1 pg to 1.0 ng per ml of TPA induces subculturability in 10 to 50% of the cultures. Control cultures repeatedly exposed to 0.2% DMSO could be subcultured at most 2 to 3 times in accordance with previous experiments with primary epithelial cell cultures (14).

Based on earlier studies of MNNG-exposed primary cell cultures (8), 0.1 μg of MNNG per ml was chosen as an initiating dose. From the above results, it was apparent that 10 pg of TPA per ml would be an optimal concentration to use. This concentration induced cell line status in one-third of the exposed cultures (Table 1). TPA exposure period was chosen to be from Day 6 (early cell colony stage) to Day 30 (postconfluent stage). Twice-
Table 1

Effect of TPA on the subculturability of tracheal epithelial cells

<table>
<thead>
<tr>
<th>TPA (pg/ml)</th>
<th>No. of cultures surviving subculturing/ no. of cultures tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>10^-3</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>10^-2</td>
<td>1/8 (13)</td>
</tr>
<tr>
<td>10^-1</td>
<td>3/8 (38)</td>
</tr>
<tr>
<td>10^-0</td>
<td>3/6 (50)</td>
</tr>
<tr>
<td>10^0</td>
<td>0/8 (0)</td>
</tr>
</tbody>
</table>

weekly exposures coincided with the twice-weekly media changes. The total number of TPA exposures was seven. The protocol is shown on Chart 1, and the end points were measured as described in the following sections.

Effect of TPA on Cell Number and CFE of Initiated Primary RTE Cell Cultures. At Day 40, all primary cell cultures were dissociated, and the number of viable cells per culture was determined. The same experiment was performed twice using 0.1 μg of MNNG per ml and 10 pg of TPA per ml. Control cultures yielded approximately 10^6 cells/culture upon dissociation (Table 2) which is typical for most studies carried out in the past. Carcinogen-treated cultures contained instead approximately 10^6 cells/culture. TPA approximately doubled the cell number in uninitiated cultures but had no measurable effect on the cell number in initiated cultures.

The CFEs of cells obtained from treated and untreated cultures were measured over a range of cell seeding densities. As Chart 4, A and B, shows, that was necessary in order to find the proper range of seeding densities in which the cells obtained from the different sets of primary cultures had a chance to express their colony-forming ability optimally. Maximum CFE was obtained with seeding densities between approximately 3 x 10^2 and 6 x 10^3 cells/sq cm. In both studies, the maximum CFE of DMSO-treated control cultures, determined on Day 40, was 0.1% or less. The maximum CFE of TPA- or MNNG-treated cultures was 0.4 to 0.6%. In contrast, maximum CFE of cultures treated sequentially with MNNG as well as TPA was 0.9% in one experiment and 1.9% when the same experiment was repeated.

Thus, the MNNG and TPA effects were at least additive and possibly more than additive.

Effect of TPA Treatment on the Subculturability of RTE Cell Cultures. As outlined in Chart 1, all cell cultures were dissociated and subcultured on Day 40. On Day 90, the frequency of cultures surviving subculturing at least 5 times was assessed. There were 18 to 24 cultures in each group. The results of the experiment can be summarized as follows. Approximately 30% of the DMSO-control cultures survived to 90 days, while 40 to 60% of the cultures exposed to either MNNG or TPA alone survived. Sequential exposure to MNNG and TPA increased the frequency of 90-day survival to 78%. It was noted that the majority of DMSO control cultures surviving to Day 90 (i.e., 5 passages) senesced between Days 90 and 120 (only 1 of 5 survived), while very few of the cultures treated with either MNNG or TPA or the combination of the 2 agents, ceased to grow.

Table 2

Effect of TPA treatment on the number of viable cells in initiated RTE cell cultures

<table>
<thead>
<tr>
<th>MNNG (μg/ml)</th>
<th>No TPA</th>
<th>TPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.17 ± 0.14</td>
<td>0.44 ± 0.32</td>
</tr>
<tr>
<td>0.1</td>
<td>1.18 ± 0.52</td>
<td>1.06 ± 0.67</td>
</tr>
</tbody>
</table>

*p Mean ± S.D.*
Effect of TPA on the Induction of Anchorage-Independent Growth in RTE Cell Cultures

At 60 days (passages 2 to 3) and again at 120 days (passages 11 to 12), all surviving cultures were tested for anchorage-independent growth (Table 3). Over 50% (Day 60) and 65% (Day 120) of the cultures first treated with MNNG and subsequently with TPA had become anchorage-independent while this had occurred only in 3% (Day 60) and 20% (Day 120), respectively, of the cultures first treated either with MNNG only or TPA only (no control cultures were ag*). In Table 4, CFE in agarose observed in this study is summarized, which shows some interesting trends. TPA treatment does induce anchorage-independent cells in a few cultures (Table 3), but the CFE in agarose is low, ~3 × 10^-5, and increases only slightly to ~5 × 10^-6 over the subsequent 60 days of culture. In the group treated with MNNG, there are fewer than 10^-6 detectable anchorage-independent cells at 60 days, and at 120 days, CFE in agarose is still only ~5 × 10^-6. In the cultures treated with MNNG and TPA, however, CFE in agarose is approximately 1 × 10^-5 cells on Day 60, and it more than triples over the next 60 days.

The effects of TPA on the appearance of the anchorage-independent phenotype were essentially confirmed in a similar study (data not shown) in which lower MNNG and TPA concentrations were used (0.01 µg/ml of MNNG and 0.001 µg/ml of TPA). At 60 days, only 1 of 52 cultures formed colonies in agarose (all groups combined). At 120 days, 50% of the cultures treated sequentially with MNNG and TPA acquired anchorage-independent growth, while only 12% of the cultures treated either with MNNG or with TPA formed colonies in agarose. Thus, the promotional effect of TPA was confirmed in this experiment, even though the anchorage-independent phenotype developed later. This is probably related to the lower initiator or promoter concentrations used in this experiment.

DISCUSSION

The purpose of our studies was to determine whether uninitiated and initiated RTE cells differ in their responsiveness to the growth-stimulatory effects of TPA and which stages of neoplastic transformation can be promoted. From previous studies, we knew: (a) that growth and replication of RTE cells can be stimulated by TPA (2, 15); (b) that the frequency of the early transformants is not affected by TPA (6, 18); (c) that the time of appearance and the frequency of the late phenotypic variants, namely, the anchorage-independent growth variants, are probably enhanced (15, 18); and (d) that the development of the neoplastic phenotype can be enhanced by TPA (15, 18, 21).

Earlier studies with tracheal organ cultures showed that the most pronounced stimulation of growth in the epithelial lining can be induced by short rather than continuous exposures to TPA (12). In the present experiments, we also found this to be true for primary RTE monolayer cultures. The CFE of freshly isolated RTE cells was not significantly affected by continuous TPA exposure (0.01 to 1.0 µg per ml of TPA) starting 24 hr after plating. In contrast, repeated, short-pulse exposures, which had also been used in the previous organ culture studies (15), were effective at extremely low concentrations (0.1 pg to 1.0 pg per ml of TPA) in enhancing growth as measured by the number of cells in late primary cultures (Chart 2), as indicated by the number and frequency of colony-forming cells in these cultures (Chart 4, A and B), and by the induction of subculturability (Table 1). The fact that these effects were seen in several independent experiments conducted over a period of about one year virtually eliminates the possibility that these are "chance findings" or that they are due to some experimental error. We have not as yet attempted to optimize the number of exposures needed to maximize the effect, nor determined the minimum number of exposures necessary to induce such effects. We are not aware of any studies in which biological effects have been detected at such low TPA concentrations. In view of this stimulation of growth of RTE cells by TPA, the growth-inhibitory effect of tumor promoters on human keratinocyte cultures reported by Parkinson and Emerson (9) is intriguing. Whether the differences between their and our observations are an indication of differences in biological responsiveness of different cell types or whether they are related to differences in TPA exposure modalities is presently uncertain but warrants further investigation.

Few reports exist in the literature describing enhancement of transformation associated events by TPA in epithelial transformation systems. Knowles (2) and later Wigley (22) described enhancement of appearance and growth of dimethylbenzanthracene induced "preneoplastic" foci in TPA-treated salivary gland cell cultures. In our own studies, the most unambiguous effect of TPA in terms of enhancement of transformation was the enhancement of the ag* phenotype which confirms previous observations in tracheal organ culture studies (15). Since the appearance of this phenotype is almost always a late development in transformation of RTE regardless of whether transformation is affected in vivo (7, 19), in organ culture (14) or in cell culture (8), it was of interest to determine whether any other effect could be measured in the cultures prior to the appearance of the anchorage-independent phenotype. As mentioned above,

TPA did not increase the frequency of RTE foci, which are the early transformants in the RTE cells system (6). The possibility had to be considered that the composition of cultures containing such cellular transformants might be changed. Therefore, the concentration of colony-forming units in the cell population was assessed (Chart 4, A and B) and was found to be significantly greater in cultures sequentially exposed to MNNG and TPA than in culture exposed to either agent alone. It is possible that the combined treatment of MNNG and TPA induced the appearance of cells which no longer require (or require less) autocrine growth factors for growth at lower seeding densities than control cells. Control cells which only formed colonies at higher seeding densities may require more such autocrine growth factors for replication. It seems reasonable to assume that such an increased concentration of colony-forming cells results in increased cell replication (not necessarily increased cell number) and thereby an increased chance for new cell variants to be generated. This might explain the greater frequency of cultures with anchorage-independent cells following initiation-promotion treatment.

The hypothesis that tumor promotion is a result of selection of initiated stem cells [selective growth stimulation of initiated cells and induction of terminal differentiation of uninhibited cells (e.g., Ref. 24)] is very attractive and available data seem to support it. Our own studies did not provide evidence for such selective stimulation of stem cells by TPA (the colony-forming cells might be considered as the stem cells of the RTE cell cultures). However, treatment of initiated tracheal cells with TPA did result in an accelerated appearance of the anchorage-independent phenotype, and the proportion of cultures showing the anchorage-independent phenotype was greatly increased. Thus, postinitiation treatment of RTE cells with TPA did result in the enhancement of a late stage of neoplastic transformation. It remains to be determined whether exposure of the anchorage-dependent cell variant and of the anchorage-independent cell variant to TPA can bring about more rapid progression to subsequent stages of transformation and the emergence of the neoplastic phenotype.

ACKNOWLEDGMENTS

The authors wish to thank Alma Gonzalez for the preparation of this manuscript.

REFERENCES

Enhanced Induction of the Anchorage-independent Phenotype in Initiated Rat Tracheal Epithelial Cell Cultures by the Tumor Promoter 12-O-Tetradecanoylphorbol-13-acetate

Vernon E. Steele, Diane K. Beeman and Paul Nettesheim


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/44/11/5068

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.