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

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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 per 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 pg/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, 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 RTE 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 agaropectin (type II) were purchased from Sigma Chemical Co., St. Louis, MO. TPA was prepared by Chemicals for Cancer Research, Eden Prairie, 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, 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...

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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° 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°. The cells were collected, and an equal volume of 20% newborn calf serum in Hanks’ balanced salt solution was added at 4°. 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 multicell (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.

Subcultureability Measurement. Primary cultures were subcultured on Day 40 by transferring 10³ viable cells into an uncoated 35-mm dish. 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.

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Charts. Effect of TPA on cell survival and growth at Day 30. Seven 1-hr exposures were given to cell cultures from Day 6 to Day 30 at which time all cultures were dissociated and counted. Column, mean cell number per dish; •, individual cell counts; bars, S.D.

Table 1
Effect of TPA on the subculturability of tracheal epithelial cells

Cell cultures were exposed to various concentrations of TPA in serum-free medium for 1 hr twice weekly from Day 6 of culture to Day 30. The primary cultures were dissociated on Day 30 and subcultured at least 5 times by Day 90.

<table>
<thead>
<tr>
<th>TPA (pg/ml)</th>
<th>No. of cultures surviving subculturing/no. of cultures tested (%)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>10^-5</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>10^-4</td>
<td>1/8 (13)</td>
</tr>
<tr>
<td>10^-3</td>
<td>3/8 (33)</td>
</tr>
<tr>
<td>10^-2</td>
<td>3/6 (50)</td>
</tr>
<tr>
<td>10^-1</td>
<td>0/6 (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 10^3 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

Primary RTE cell cultures were initiated with MNNG (0.1 μg/ml) and subsequently treated repeatedly with TPA (10 pg/ml; see Chart 1). At Day 40 of the experiment, the cultures were dissociated, and the number of viable cells per culture was determined. Control cultures were exposed to 0.2% DMSO. Means obtained were from 18 to 22 separate cultures. All treated groups have mean cell numbers significantly higher (p < 0.05; Student's t test) than control groups. TPA did not significantly increase the mean cell number in MNNG-treated groups. Treatment with MNNG, with or without TPA, significantly increased cell numbers (p < 0.001; Student's t test) over cultures treated with TPA alone.

<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>1.10 ± 0.52</td>
<td>1.06 ± 0.67</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
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\(^2\) (12, 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 \(\mu\text{g}\) 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 ng 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 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.

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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 combined cultures treated with either MNNG only or TPA only (no control cultures were ag\(^+\)). In Table 4, CFE in agar 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 agar is low, \(-3 \times 10^{-5}\), and increases only slightly to \(-5 \times 10^{-6}\) 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 agar is still only \(-5 \times 10^{-6}\). In the cultures treated with MNNG and TPA, however, CFE in agar is approximately \(1 \times 10^{-4}\) 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 \(\mu\text{g}\)/ml of MNNG and 0.001 pg/ml of TPA). At 60 days, only 1 of 52 cultures formed colonies in agar (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.

**Table 3**

<table>
<thead>
<tr>
<th>MNNG ((\mu\text{g/ml}))</th>
<th>Day 60</th>
<th>Day 120</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0/7*</td>
<td>1/15</td>
</tr>
<tr>
<td>0.1</td>
<td>0/11</td>
<td>7/13</td>
</tr>
</tbody>
</table>

* Number of primary cultures positive for anchorage growth independent per number of cultures tested. All cultures surviving to Days 60 and 120, respectively, were tested.

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<table>
<thead>
<tr>
<th>MNNG ((\mu\text{g/ml}))</th>
<th>No TPA</th>
<th>TPA</th>
<th>No TPA</th>
<th>TPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.9</td>
<td>1</td>
<td>5</td>
<td>3.8</td>
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**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\(^2\) (12, 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).

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ACKNOWLEDGMENTS

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