Tumor Promotion Studies in Rat Tracheal Epithelium

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The tracheal epithelium of the Fischer 344 rat is histologically very similar to that of the human bronchus. Also, carcinomas of tracheal origin in F-344 rats are similar in morphology to human bronchogenic carcinomas. Tumor promotion in rat tracheal epithelium was studied by using two model systems.

The first is a heterotopic transplant system in which rat tracheas are implanted subcutaneously on the backs of isogenic recipients. In the first system, the epithelium was topically exposed to pellets containing 7,12-dimethylbenz(a)anthracene (DMBA), used as the initiating agent, followed by pellets containing the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), the promoting agent. After 98 weeks, a three- to fourfold increase in the percentage of tracheas having malignant tumors was seen in tracheal transplants receiving both DMBA and TPA compared to DMBA alone. Exposure of the tracheal grafts to TPA alone resulted in epithelial hyperplasia and inflammation, but no dysplastic lesions.

The second system is an organ culture-cell culture system in which small pieces of trachea are grown in organ culture, then epithelial cells are grown from these pieces as primary cell cultures. The organ cultures were exposed to the direct alkylating agent, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) used as the initiator, then multiple short exposures to TPA were used to promote. Primary cell cultures and cell lines were then established from these explants. After 52 weeks, a fivefold increase in the percentage of explants producing tumorigenic cell lines was observed when MNNG + TPA-exposed explants were compared to MNNG-exposed explants. Tracheal explants exposed to TPA alone produced many cell lines but none tested were tumorigenic. These two systems provide a means to study tumor promotion in respiratory epithelium. The evidence more importantly suggests that airborne promoting substances may play a key role in the development of bronchogenic carcinoma.

Introduction

The epithelial mucosa lining the human respiratory tract is in contact with a wide variety of environmental pollutants. These pollutants are chiefly made up of particulates and/or gases. Epidemiological evidence suggests with some degree of certainty that lung cancer in humans is a multistage process (1-3). It is extremely difficult to separate and understand the interacting, synergistic and promoting effects of toxic agents in humans since man is continuously exposed to complex mixtures such as cigarette smoke and automobile exhaust. To delineate these processes and understand them, animal and cell culture models using respiratory tract epithelium have been developed. To develop these systems for the study of tumor promotion in the respiratory tract, certain assumptions and simplifications have been made. Rat tracheal epithelium was chosen because it is histologically similar to human bronchial epithelium; the tumors arising from this tissue are similar in morphology to human bronchogenic carcinomas; and there is an extremely low background incidence of spontaneous respiratory tract tumors in specific pathogen-free F-344 rats. For simplification, single initiators and single promoters were used. The initiators employed here are known carcinogens, either direct acting or requiring metabolic activation. The promoter used was the phorbol diester tumor promoter proven most active.
in mouse skin, 12-O-tetradecanoyl-phorbol-13-acetate or TPA (4).

To demonstrate the phenomenon of tumor promotion in tracheal epithelium, the heterotopic tracheal transplant system was utilized (5). The qualitative and quantitative aspects of the tumor response have been previously established for the transplant system (6). Other studies have developed methodology for the controlled release and accurate dosing of carcinogens and phorbol esters to the tracheal mucosa (7). By using this system, crystalline asbestos and nickel subsulfide have been shown to enhance the tumor frequency of DMBA-initiated tracheal cells (8, 9). Cell culture models using similar tissue have also been developed. Previous studies have shown that in vitro exposed tracheal epithelium could be transformed in a dose-dependent manner (10). The tumors formed following the inoculation of transformed epithelial cell lines were similar in morphology to those induced in vivo but appeared in a much shorter time span. A preliminary set of experiments also showed that the mucociliary epithelium of cultured tracheal explants responds to phorbol ester exposure by a dramatic increase in cell proliferation (11, 12). The kinetics of DNA synthesis in the cultured rat tracheal epithelium was very similar to that of mouse skin. The extent of stimulation was dependent on the duration of exposure, the concentration of TPA, and the serum level of the medium. These findings led to the design of the initiation promotion experiments described here.

Materials and Methods

Tracheal Transplants

Freshly excised tracheas from female animals were sutured onto a slightly longer length of semi-rigid polyethylene tubing. After the tracheal grafts had established, pellets, prepared as described below, were surgically placed into the tracheal lumen and the openings were closed with silk suture material. Two tracheas per host were implanted subcutaneously to the retroscapular region as described previously (5).

Pellets for Chemical Exposure

The carcinogen initiator used was 7,12-dimethylbenz[a]anthracene (DMBA) which was purchased from Eastman Kodak Co. (Rochester, NY) and recrystallized before use. The tumor promoting agent was 12-O-tetradecanoyl-phorbol-13-acetate (TPA) from Dr. Peter Borchert (University of Minnesota, Minneapolis, MN). The DMBA and TPA were dissolved in melted beeswax and molded into cylindrical pellets. The procedures for making pellets has been described previously (5, 7). The final concentrations of DMBA or TPA in the pellets were determined by ultraviolet spectrophotometry.

Experimental Design

Tracheal transplants were first either exposed to pellets containing DMBA or blank beeswax pellets. After 4 weeks the DMBA pellets were surgically removed and pellets containing TPA or blank beeswax were inserted. Similarly, the beeswax pellets were replaced with pellets containing TPA. Hence, all transplants underwent the same number of surgical manipulations and all contained pellets throughout the experiment. The transplanted tracheas were palpated biweekly for tumor appearance. Tracheas with tumors 2 cm in diameter were removed from the experiments. All remaining tracheas were harvested after 98 weeks and histologically examined for lesions resulting from chemical exposure.

Organ Culture

Tracheas were excised from Fischer 344 female rats and cut into explants, 2 × 3 mm, as described by Marchok et al. (13). The explants were placed in organ culture dishes (Falcon Plastics, Oxnard, CA) on Gelman TCM filter paper (Gelman Instruments Co., Ann Arbor, MI) supported by a stainless steel grid.

Chemical Exposures

The direct acting carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), from Starks Inc. (Buffalo, NY), was used as the initiating agent. Exposure of the organ cultures lasted for 3 hr on days 3 and 6. The TPA was prepared as described previously (11). Three days after the last carcinogen exposure the explants were exposed for 1 hr to either 0 or 1.0 μg TPA/mL medium every 6 days for 3 weeks. The exposures were performed in serum-free Waymouth's medium with the dimethylsulfoxide solvent concentration at 0.2%. Control cultures received 0.2% dimethyl sulfoxide alone.

Cell Culture

Following the last TPA exposure, the explants were placed on the bottom of organ culture dishes to stimulate the outgrowth of epithelial cells. The explants were replanted weekly for 10 weeks to maximize the number of epithelial cells reaching the surface of the dish. Epithelial cell lines were established from primary cultures after the appearance of morphologically altered cells. The cultures and
subcultures were passaged by using 0.2% trypsin-EDTA (Grand Island Biological Co., Grand Island, NY).

**Assay for Tumorigenicity**

Epithelial cell lines which reached the fifth passage at certain times after exposure were inoculated into immunosuppressed isogenic rats as described previously. Four inoculations of 10⁶ cells each were placed intramuscularly into the hind legs of two animals at each time point. The legs were palpated twice weekly for the appearance of tumors. The appearance of one tumor from four inoculations was considered a positive test. The animals were observed for 100 days and the tumors were histologically examined to verify tumor type.

**Primary Cell Culture Exposures**

Tracheal epithelial cells were enzymatically removed from male F-344 rat tracheas as described by Wu and Smith (15). The cells were plated on collagen-coated tissue culture dishes (15). Attached cells were exposed to carcinogen in serum-free medium beginning on day 1 for 3 hr and at various days thereafter. All cell counts were done by hemocytometer. Subculturing was performed by using 0.2% trypsin-EDTA.

**Results**

**Heterotopic Tracheal Transplant System**

Over the 4-week period that the 200 μg DMBA pellets were inside the tracheal graft, an average of 188 μg of DMBA was released per trachea. The 100 μg TPA pellets released over 80 μg of TPA in the first 4 months (approximately 1.1 μg/day for the first 2 months and 0.3 μg/day the second 2 months). It is assumed that nearly all 100 μg of TPA was delivered if the pellet remained in the full 94 weeks of the experiment. Of the 20 tracheas exposed only to 188 μg DMBA (no TPA), only three developed palpable invasive carcinomas and three more with a microinvasive carcinoma (Table 1). If the tracheas were only exposed to the TPA pellets (no DMBA) then no tumors formed. Combining these two agents in 22 tracheas yielded 13 tracheas with palpable invasive carcinomas and three more with noninvasive exophytic carcinomas growing into the tracheal lumen. Previous studies have shown that no tumors are found in tracheas receiving blank beeswax pellets for this time period (6).

Tracheas not developing tumors were examined histologically. In the DMBA + TPA group, all of the remaining tracheas contained one or more metastatic-dysplastic lesion(s). Of tracheas exposed to DMBA alone and not developing tumors, five of the 16 had lesions, while none of the tracheas in the TPA only group had lesions.

The effect of TPA on DMBA exposed tracheas was not only that of increasing the frequency of tumors, but that the tumors appeared much earlier. The first tumor in the DMBA + TPA group reached 2 cm in diameter at 57 weeks while the first tumor in the DMBA alone group took 80 weeks, nearly 6 months longer. Overall, 10 of the 13 palpable tumors in the DMBA + TPA group had reached 2 cm in diameter and were removed before the first tumor appeared in the DMBA only group. In a second set of experiments by Klein-Szanto (17), also summarized in Table 1, transplants were exposed to pellets containing 100 μg DMBA of which 35 μg was delivered during a 2-week exposure period. In 38 tracheas exposed only to 35 μg DMBA, only 2 developed carcinomas by 86 weeks. In contrast, if the DMBA pellets were followed by 100 μg TPA pellets, 19 of the 56 tracheal transplants or 34% developed tumors. The first tumor in the DMBA + TPA group reached 2 cm at approximately 43 weeks, while the first tumor in the DMBA alone group reached equal size at 47 weeks. Results similar to DMBA + beeswax were recorded if no second pellet was implanted.

**Organ Culture-Cell Culture System**

A schematic diagram of the organ culture-cell culture system is shown in Figure 1. Since previous studies had shown that a concentration of 0.001 μg MNNG/mL produced tumorigenic cell lines (10), a 10-fold lower concentration, 0.1 ng MNNG/mL, was expected to minimize the transformation frequency.
The TPA concentration and exposure duration were chosen since previous studies had shown that under these conditions there was a maximal increase in cell proliferation (12). Three major events were scored during the course of the experiment in terms of time of occurrence after MNNG exposure and frequency: (1) the appearance of morphologically altered cells, (2) the establishment of cell lines and (3) the development of oncogenicity. Table 2 shows the time and frequency of the establishment of cell lines from morphologically altered cells. As we had observed previously (18), morphologically altered cells appeared in primary cell cultures between 3 and 7 months following carcinogen exposure. These cells had a markedly higher nuclear-to-cytoplasmic ratio, were smaller and remained
tightly packed as they grew rapidly across the surface of the dish. Unexpectedly, TPA exposure also induced the appearance of these cells and subsequently we were able to subculture continuously proliferating cell lines (11). There were no gross morphological differences between carcinogen induced or TPA induced morphologically altered cells or cell lines. An example of one such cell line is shown in Figure 2. Primary cell cultures not containing morphologically altered cells were maintained for up to 7 months, but subculture of unaltered cells was unsuccessful. No morphologically altered cells were seen in solvent control cultures. In cultures derived from MNNG + TPA treated explants, morphologically altered cells appeared at about 132 days, significantly sooner \( (p<0.05) \) than in primary cultures derived from explants exposed to only MNNG which was 209 days. When the altered-cell patches reached 2-3 cm in diameter, attempts were made to subculture the cells. Future subcultures were made at confluency. The time required for the cells to grow to confluency four times from the first subculture to the fifth subculture was markedly shortened in the MNNG + TPA group compared to the MNNG only or TPA only group. An average of only 34 days was needed in the MNNG + TPA group, while 73 days were needed in the MNNG only group, and 74 days in the TPA only group. This acceleration of growth was accompanied by an acceleration of tumorigenic potential. Of the five cell lines tested before passage 9 in the MNNG + group, three were tumorigenic. In contrast, of the eight cell lines tested before passage 9 in the MNNG-only group, none was tumorigenic. In order to assess a true acceleration of tumor formation due to TPA exposure, the cell lines were inoculated at various times up to 1 year after carcinogen exposure. Table 3 shows that no tumors resulted from the inoculations of the 14 cell lines derived from explants exposed to TPA alone. Of the ten cell lines derived from explants exposed only to MNNG only three were tumorigenic at 365 days and neither of the two cell lines that had been established by 310 days were tumorigenic. Cell lines derived from explants receiving MNNG + TPA were established as early as 150 days, and six of the eight tumorigenic cell lines were tumorigenic at 310 days and earlier.

The tumors were excised when they reached 2 to 3 cm in diameter and examined histologically. All

Table 3. Frequency of tumorigenic cell lines at various times after exposure to MNNG and/or TPA.

| Times after first MNNG exposure, days | Frequency of tumorigenic cell lines (no. tumorigenic/no. inoculated) \( ^{b} \) |
|--------------------------------------|-------------------------------------------------|
|                                      | TPA     | MNNG    | MNNG + TPA         |
| 100                                  | 0/0     | 0/0     | 0/0               |
| 150                                  | 0/0     | 0/0     | 1/1               |
| 200                                  | 0/0     | 0/0     | 3/3               |
| 250                                  | 0/2     | 0/0     | 3/4               |
| 310                                  | 0/6     | 0/2     | 6/7               |
| 365                                  | 0/14    | 3/10    | 8/11              |

\( ^{a} \) Modified from Steele et al. (19).

\( ^{b} \) The tumors were all differentiated carcinomas. The number inoculated also represents the total number of lines which had reached the fifth passage or more by the indicated timepoint.

![Figure 2](image)

**Figure 2.** Typical tracheal epithelial cell line derived from explants exposed to MNNG and/or TPA. Phase contrast, \( \times \) 150.
tumors were either squamous cell carcinomas or adenosquamous cell carcinomas (Figs. 3 and 4). The time between inoculation and tumor appearance did not appear to be shorter for the tumorigenic cell lines in the MNNG + TPA group compared to the tumorigenic cell lines on the MNNG only group. Also the growth rate of the tumors once palpable appeared equal in the two groups.

Correlation of Growth Promotion and Tumor Promoting Activity

The ability to enhance in vitro growth seems to be a common property of carcinogens and the tumor promoter TPA. To determine if this phenomenon relates to tumor promotion we asked the following questions. Does the enhancement of growth in cul-
tured tracheal epithelium correlate with the ability of various compounds to promote mouse skin carcinogenesis? Using the organ culture-cell culture system we exposed explants to various compounds as shown in Table 4 and assayed their ability to induce cell lines. Four 1-hr exposures to mezerine, a non-promoting TPA analog, and acetic acid induced no cell lines. A very low percentage of cell lines was established from DMSO, phorbol, and 40-methyl TPA-exposed explants. Four exposures to TPA at 1 or 0.01 µg/mL produced a high percentage of cell lines. A single 1-hr exposure to 1 µg TPA/mL was not nearly as effective as multiple exposures.

Enhancement of Growth Using Primary Tracheal Epithelial Cells

When 10⁴ freshly isolated tracheal cells are plated in a collagen-coated 60 mm dish, they grow and reach confluency at 10–14 days, then senescence and evert from the dish. Single or multiple MNNG exposures can cause a dramatic growth alteration in cells of similar cultures. These cells escape the senescence process and become established cell lines. Table 5 shows that increasing numbers of cells are present at day 30 as the number of exposure to MNNG increased. Attempts to establish cell lines from these cultures were more successful after multiple MNNG exposures than with a single exposure. Six or eight MNNG exposures was the most efficient.

In preliminary experiments we found that TPA can also induce cell lines following multiple exposures of primary epithelial cell cultures (data not shown). From these data we have designed an initiation-promotion scheme consisting of a single low dose of MNNG given on day 1 of culture followed by multiple TPA exposures between days 6 and 30. The single low dose of MNNG (0.01 µg/mL) produced no cell lines in previous studies (Pai et al., unpublished data). The TPA concentrations chosen either induce few or no cell lines. The objective of these experiments is to determine if initiated cells are more susceptible to growth alteration than non-initiated cells, and to develop a rapid assay system for promoting compounds of the respiratory tract.

Discussion

The in vivo and in vitro experiments described here demonstrate that two-stage carcinogenesis indeed occurs in respiratory tract epithelium. Following the initiation of tracheal cells by a chemical carcinogen, exposure to a known tumor promoting substance both increases the frequency of final tumor yield and decreases the latency period to tumor appearance in animals and in cultured cells. Exposure of respiratory epithelial cells to a tumor promoter alone causes a reversible hyperplasia in vivo and a permanent loss of growth control in cultured cells. This loss of growth control in cultured cells correlates with the promoting potential of various agents as measured in the mouse skin model. If cells are exposed only to initiating doses of carcinogen, many preneoplastic changes occur but few are expressed.

Promoting agents probably act by causing a loss of growth control in both initiated and non-initiated cells. Promoters also cause a loss of cellular commitment to terminal differentiation (20, 21). The loss of growth control and altered cell differentiation may or may not be independent events. The fact that TPA-induced cell lines which have altered growth control can differentiate normally when placed back in vivo (11) suggests that the two pathways are independent. The important fact may be that in non-initiated cells in vivo the effects of promoter exposure are transient, while in initiated cells the effects are permanent and heritable. Since many of the ex-

| Chemical       | No. of exposures | Concentration, µg/mL | No. of cell lines per no. exposed explants | %   |
|----------------|------------------|----------------------|------------------------------------------|-----|
| DMSO           | 4                | 0.2%                 | 1/15²                                    | 7   |
| Mezerine       | 4                | 0.2%                 | 0/3                                      | 0   |
| Acetic Acid    | 4                | 0.012%               | 0/8                                      | 0   |
| Phorbol        | 4                | 0.012%               | 2/12                                     | 17  |
| 40-MeTPA       | 4                | 0.1%                 | 1/9                                      | 11  |
| TPA            | 4                | 0.1%                 | 6/8⁴                                     | 75  |
| TPA            | 4                | 1.0%                 | 2/8                                      | 25  |
| TPA            | 4                | 5.0%                 | 5/5                                      | 100 |

*Combined results of two experiments.

| No. of MNNG exposures | No. of cells (± S.D.) x 10⁴ |
|-----------------------|-----------------------------|
|                       | At day 18⁴                   | At day 30⁵                   |
| 0                     | 0.04 ± 0.03                 | 0.02 ± 0.02                 |
| 1                     | 0.61 ± 0.51                 | 4.59 ± 4.74                 |
| 2                     | 2.21 ± 0.91                 | 9.76 ± 4.69                 |
| 4                     | 5.29 ± 3.44                 | 18.23 ± 6.78                |
| 6                     | 6.42 ± 3.69                 | 18.01 ± 17.97               |
| 8                     | 4.84 ± 1.69                 | 22.36 ± 12.23               |

⁴Cells were exposed to 0.1 µg MNNG/mL for 3 hr in serum-free medium on days 1, 3, 5, 7, 10, 12, 14, and 17 (one exposure on day 1, 2 exposures on days 1 and 3, etc.).

⁵Cell numbers were indirectly determined by estimating cell density times X area covered by the cells.

*Cell number was determined directly by dissociation of the cultures and counting the cells using a hemacytometer.
logenous growth-controlling factors in whole animals are not present under culture conditions, cells exposed to TPA in cultural lose growth control and never regain it until they are placed back in vivo.

Tracheal primary cultures appear to be a very useful for quantitative studies of the effects of TPA on initiated and non-initiated cells. If initiated cells are more susceptible to growth alteration than non-initiated cells, then initiated cells should preferentially grow under conditions where noninitiated cells would not. Since alterations in growth can be detected as early as 30 days, the primary culture system may be a rapid, useful tool to assay for suspected tumor promoting agents.

Several other studies also suggest that promotion is an important mechanism in respiratory carcinogenesis. Using the heterotopic tracheal transplant system, asbestos and nickel subsulfide were shown to enhance the tumor response in DMBA-initiated tracheal transplants (\(\phi\)). Additionally, alveologenic tumors in mice can be promoted by systemic administration of phorbol or butylated hydroxytoluene as presented by Witschi elsewhere in this volume.

The mechanisms by which TPA can alter the frequency of tumor induction and accelerate tumor formation are unknown. The tracheal transplant and cell culture systems described here provide a means to study tumor promotion and identify substances which have a potential to promote respiratory cancer.

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