Promotion-like Enhancement of Tracheal Carcinogenesis in Rats by 12-O-Tetradecanoylphorbol-13-acetate

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ABSTRACT

Experiments were conducted to determine whether two-stage carcinogenesis could be observed in rat tracheal epithelium using 7,12-dimethylbenz(a)anthracene (DMBA) as initiator and 12-O-tetradecanoylphorbol-13-acetate (TPA) as promoter. Heterotopic tracheal transplants in Fischer 344 rats were first exposed to 188 μg of DMBA delivered over a four-week period and subsequently to 100 μg of TPA. TPA was released from beeswax pellets at a rate of 1.1 μg/day during the first two months and at a rate of 0.3 μg/day for the subsequent two months. TPA alone caused marked inflammation and epithelial hyperplasia in tracheal grafts but no metaplastic or dysplastic changes. The tumor incidence in tracheas exposed to DMBA only was 20%; that in tracheas exposed to DMBA followed by TPA was 72%. TPA also accelerated the appearance of tumors. The mean tumor induction time in the group exposed to DMBA only was 91 weeks as compared to 75 weeks in the group exposed to DMBA and TPA. The data indicate that TPA enhances the tracheal tumor response in a manner similar to that of tumor promotion in mouse skin.

INTRODUCTION

The phenomenon of multi- or 2-stage carcinogenesis has been most clearly established in mouse skin (2). However, in recent years, it has been shown to occur also in other tissues such as the liver (9), the urinary bladder (3), and the lung (1). The target tissue involved in the latter case is the alveolar epithelium where most of the pulmonary adenomas and adenocarcinomas are of alveolar origin. Recently, in vitro studies carried out in our laboratory (11, 12) suggested that it is possible to produce a promotion-like effect in rat tracheal epithelium initiated with N-methyl-N-nitro-N-nitrosoguanidine using TPA. The investigations reported here were carried out to determine whether 2-stage carcinogenesis could be demonstrated in vivo in the mucosa of the conducting airways. DMBA was used as the initiation agent, and the phorbol ester TPA was the promoting agent. The experimental system utilized was the heterotopic tracheal transplant model in which rat tracheas transplanted s.c. to the dorsum of isogenic recipients are exposed by topical application to the initiating and promoting agents. A series of previously published studies has established the qualitative and quantitative aspects of the tumor response in this experimental model (6, 7, 14, 15) and the methodology for carcinogen and phorbol ester delivery to the tracheal mucosa (8).

MATERIALS AND METHODS

Pellets for Delivery of Chemicals. DMBA was obtained from Eastman Kodak Co., Rochester, N. Y. and was recrystallized before use. TPA (both tritiated and nonradioactive) was obtained from Dr. Peter Borcherdt (University of Minnesota, Minneapolis, Minn.). The TPA and DMBA were dissolved in melted beeswax (laboratory grade; Fisher Scientific Co., Fairlawn, N. J.). Cylindrical pellets were formed by the use of a stainless steel pellet maker. The procedures have previously been described (5, 8, 17). Pellets containing either 200 μg of DMBA or 100 μg of TPA were produced. The concentrations of the DMBA and TPA were confirmed by UV spectrophotometry. The molar extinction coefficients used for DMBA were: ε₂₅₀ (79,000) in benzene and TPA and ε₂₅₀ (3,793) in cyclohexane. The TPA concentration was further confirmed by counting aliquots of dissolved pellets containing labeled TPA in a Beckman LS-350 scintillation counter.

Animals and Tracheal Transplants. Tracheas from female Fischer 344 rats were transplanted s.c. to the retroscapular region of isogenic recipients as described before (5).

The pellets were inserted into the tracheal lumen through a small incision which was subsequently closed with silk suture material.

Experimental Design of the Tumor Promotion Study. Three exposure groups were used in this experiment. Group 1 consisted of 22 tracheas first exposed to pellets containing 200 μg of DMBA for 4 weeks. The DMBA pellets were then removed, and pellets containing 100 μg of TPA were implanted. The TPA pellets remained in place for the duration of the experiment. Group 2 consisted of 20 tracheas implanted with pellets containing 200 μg of DMBA. Four weeks later, these pellets were removed and were replaced with beeswax pellets containing neither DMBA nor TPA. Group 3 consisted of 20 tracheas implanted first with blank beeswax pellets. At 4 weeks, the blank pellets were removed and replaced by pellets containing 100 μg of TPA. Hence, all tracheas were surgically manipulated the same number of times, and all tracheas contained pellets with a matrix made of beeswax for the entire tumorigenesis study. Tracheas were palpated biweekly for the appearance of tumors. Tracheas were removed surgically when tumors 2 cm in diameter had developed or when the host animal became moribund. At 98 weeks after the start of the DMBA exposure,
all surviving animals were killed, and the tracheas were removed and processed for histology.

All tracheas were assessed histopathologically to determine type, severity, and extent of mucosal changes resulting from the various exposures. Tracheas were prepared and assessed as described previously (17). To determine the rate of release as well as the early morphological alterations caused by these chemicals, additional tracheas were exposed to either TPA alone or DMBA followed by TPA. These were collected in groups of 6 at predetermined intervals. The pellets were analyzed for the amount of residual chemical, and the tissues were examined histologically. Six tracheas each were sampled at Days 1, 3, 7, 14, and 28 and at 3 and 4 months.

RESULTS

Delivery of DMBA and TPA to Tracheal Grafts

The DMBA-containing pellets were removed from the tracheas at 4 weeks. These pellets still contained an average of 12.3 ± 3.8 µg of residual DMBA. Therefore, a mean of 188 µg of DMBA had been delivered. A study on the release rate of TPA from intraluminal pellets was conducted and is summarized in Chart 1. The TPA-beeswax pellets were removed from the grafts, rinsed with 0.9% NaCl solution to remove adhering mucus, and dried. The pellets were then dissolved in the appropriate solvents, and aliquots of the resulting solutions were quantified spectrophotometrically or, in the case of the tritiated compound, through the use of liquid scintillation techniques (see "Materials and Methods"). It can be seen that delivery of the TPA to the grafts lasted for at least 4 months in tracheas previously exposed to DMBA. The release rate of TPA from the pellets was approximately 1.1 µg/day for the first 2 months and approximately 0.3 µg/day between 2 and 4 months. In tracheas not preexposed to the carcinogen, the release rate was somewhat faster than that observed following carcinogen exposure, namely, about 1.6 µg/day during the first month. In both cases, protracted exposure of the epithelium to TPA was achieved.

Histological Appearance of Tracheal Transplants during the First 5 Months of Exposures to DMBA and/or TPA

TPA Only. Tracheas exposed to pellets containing 100 µg of TPA were characterized by inflammation and edema, granulocytic infiltration, and epithelial hyperplasia. As early as 1 and 3 days, there were granulocytes in the epithelium and a marked subepithelial histiocytic reaction. The epithelium was in general hyperplastic, but there were also small patches of necrosis. Sterile cysts were present in the tracheal wall of some grafts after 7 days of exposure, but by 14 and 28 days, the edema and inflammation had regressed considerably. At that time, most of the epithelium was mildly hyperplastic mucociliary epithelium. No stratified epithelial lesions were observed.

DMBA and DMBA Followed by TPA. Tracheas exposed to 200 µg of DMBA pellets for 4 weeks and then examined histologically were uniformly lined by keratinizing squamous metaplastic epithelium. In the tracheas exposed to DMBA first and subsequently to TPA, inflammation was conspicuous during the first few weeks. The epithelial lining was hyperplastic and/or metaplastic, and verrucous as well as papillary squamous lesions were observed. In subsequent months, the submucosa became dense, fibrotic, widespread metaplastic-dysplastic lesions developed, and the first microinvasive carcinomas were detected at 3 and 4 months. In the tracheas which received blank beeswax pellets subsequent to the DMBA exposure, the metaplastic lesions were fewer in number and less dysplastic; the submucosa was less sclerotic.

Tumorigenesis Study

The development of tracheal tumors is summarized in Chart 2. The carcinoma incidence was 73% in the tracheas exposed to DMBA followed by TPA as compared to 20% in tracheas exposed only to DMBA followed by blank beeswax pellets. The difference between the 2 groups is significant at the p = 0.02 level using a χ² test of binomial distribution corrected for continuity (10). No tumors developed in the 20 tracheas exposed to blank beeswax pellets followed by TPA. All but 4 of the carcinomas which occurred were large, highly invasive...
tumors which destroyed the trachea in which they originated. The other 4 carcinomas were either microinvasive carcinomas or noninvasive exophytic carcinomas growing into the tracheal lumen.

In addition to a larger number of tumors in the DMBA-TPA group, the tumors also began to appear earlier than in the group not promoted with TPA. The first palpable tumor in the group exposed to DMBA and TPA reached 2 cm in diameter 57 weeks after carcinogen exposure. Ten palpable squamous carcinomas were removed from animals in that group before the appearance of the first carcinoma in the group exposed to DMBA alone.

The experiment was terminated at 98 weeks since some of the animals were developing leukemias. The following tracheas were collected at this time and processed for histology: 4 tracheas in the DMBA-TPA group; 7 tracheas in the DMBA only group; and 16 tracheas in the TPA group. Table 1 summarizes the histological findings in the trachea of all groups. Of the 16 carcinomas in the DMBA-TPA group, 3 were microinvasive; the other 6 trachea showed one or more metaplastic-dysplastic lesions. In the DMBA-beeswax group, there were 3 palpable invasive carcinomas, one microinvasive carcinoma and 5 metaplastic-dysplastic lesions, and more than one-half of all tracheas had no significant lesions. In the group receiving blank beeswax pellets first followed by TPA, no carcinomas and no lesions were found in 20 tracheas.

DISCUSSION

The purpose of our study was to determine whether rat tracheal mucosa initiated in vivo by a weakly tumorigenic dose of DMBA could be promoted by topical application of TPA in a manner similar to initiated mouse skin. The experiments showed that 2-stage carcinogenesis does indeed occur in the mucosa of the conducting airways of rats. This supports our previous findings, demonstrating the initiation and promotion of rat tracheal epithelium in vitro (11, 12). Whether the observed TPA effect is indicative of a promotion or of an enhancement effect is, in our opinion, probably more a matter of semantics than of biological mechanisms.

The present studies also give support to the conclusions reached in previous experiments in which we interpreted the marked enhancement of the tracheal tumor response by asbestos as promotion (16). Thus, our in vivo and in vitro investigations strongly suggest that promotion might indeed be an important mechanism in the pathogenesis of lung cancer. The findings of Armuth and Barenblum (1) and of Witschi et al. (18), demonstrating the promotion of alveologenic tumors in mice by systemic application of phorbol and butylated hydroxytoluene, respectively, indicate that the principle similarly applies to the alveolar parenchyma.

All of the tracheas exposed sequentially to DMBA and TPA showed either dysplastic or neoplastic lesions. In contrast, only 50% of tracheas exposed to DMBA but not to TPA developed either type of lesion, and TPA-exposed tracheas developed no lesions. This lends support to conclusions derived from previous studies in which "carcinogen-altered cells" (initiated cells?) were detected and quantitated with the use of an in vitro assay (13), namely, that normally, only a fraction of the carcinogen-altered cells are able to express their tumorigenic potential in vivo. Thus, part of the carcinogen effects remains hidden unless permissive and/or promoting conditions reveal their existence.

Surprisingly, several microinvasive carcinomas were found in the serial sampling study as early as 3 months (DMBA and TPA). Yet, the first palpable carcinoma appeared only after approximately 1 year. Similar observations were also made in our earlier studies (6, 7) although these did not involve promoter. This suggests that some carcinomas develop early but, for reasons presently not understood, enter a rapid growth phase only many months later.

Together with the earlier in vitro studies (11, 12), our investigations demonstrate for the first time that tumor promotion occurs in airway mucosa of rats using classical initiating and promoting agents. They thus provide an important piece of evidence in support of the hypothesis that promoters might play a decisive role in the pathogenesis of bronchogenic carcinoma (4, 18, 19).

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REFERENCES


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