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AN ORGAN CULTURE-CELL CULTURE SYSTEM FOR STUDYING MULTISTAGE CARCINOGENESIS IN RESPIRATORY EPITHELIUM

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Respiratory Carcinogenesis in vitro

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Many studies support the hypothesis that the induction and development of cancer is a multistage process. Most of this evidence stems from the now classical initiation-promotion studies using mouse skin. These experiments have recently been summarized by Boutwell (2). At present, it is not clear whether similar processes occur during tumor development in other tissues. Recent studies, using mainly fibroblast cell lines, have demonstrated multistage oncogenesis in vitro, suggesting that this phenomenon may be a more general one (3,7,8).

In the present report we describe the development of an in vitro model system which should permit a detailed investigation of the various phases of carcinogenesis in respiratory tract epithelium. Until now, little work has been carried out using epithelial cells to study transformation in vitro because of the difficulty of maintaining epithelial cells in culture. This work has been reviewed by Weinstein et al. (11).

In our experiments the tracheal epithelium is first exposed to the carcinogen and/or promoter while in organ culture. This method is used to preserve tissue integrity and to maintain proliferation and differentiation similar to that found in the mucociliary epithelium in vivo. Then the epithelial cells are allowed to grow off the explants in order to provide rapidly-expanding primary cultures which can be maintained for at least 4 to 6 months in vitro. Cell lines can then be established from such primaries and tested for oncogenicity.

Three studies using this system are presented below. The first demonstrates neoplastic transformation of tracheal epithelium after exposure to a complete carcinogen. The second shows that exposure of the organ cultures to the promoting agent TPA (12-O-tetradecanoyl-phorbol-13-acetate) stimulates DNA synthesis. The third study, which is still in progress, is an attempt to demonstrate promotion in the organ culture-cell culture system. Before discussing these investigations, we will first briefly summarize the key features of this *in vitro* system.

The Organ Culture-Cell Culture System

Tracheal explants were made from 10-12 week-old female Fisher-344 rat tracheas as previously described by Marchok *et al*. (4). The 2 X 3 mm explants were then placed luminal side up on a 1.2 µm Gelman TCM filter which was supported by a stainless steel grid in a conventional organ culture dish. The Gelman filter paper did not permit the outgrowth of cells from the explants. Except as noted, the media used for all organ cultures consisted of Waymouth's MB 752/1 plus 0.1 µg insulin and 0.1 µg hydrocortisone/ml and 2% fetal bovine serum. Following exposure of the organ cultures to carcinogen and/or the promoting agent, the explants were placed on the bottom of tissue culture dishes. This contact with the tissue culture dish then permitted the outgrowth of cells from the explant. The dishes contained an enriched medium (Waymouth's + 10 µg insulin and 0.1 µg hydrocortisone/ml + 10% FBS + additional amino acids, sodium pyruvate, fatty acids, and putrescine), as described by Marchok *et al*. (6), for the maintenance of long-term primary epithelial cell cultures. After

approximately 7 days, the explants were replanted to produce a second outgrowth. This replanting process was repeated up to ten times to allow as many of the exposed cells to grow off the explant as possible. The cells remaining on the dish once the explant was removed were designated a "primary culture" if they survived at least 3 weeks. If these cells could be passed, after maintenance for a period of time in primary culture, and be subcultured at least five times, then they were designated "cell line cultures." Therefore, for each explant there existed the possibility of producing 10 primary cultures and 10 cell line cultures. Tumorigenicity of the cell line cultures during passage was determined by injecting 10^6 cells into the thighs of isogeneic rats which were immunosuppressed by thymectomy and x-irradiation.

Carcinogen Exposure Study

In order to study multistage carcinogenesis in respiratory epithelium in vitro, we had to determine first whether neoplastic transformation could be demonstrated. On days 3 and 6 after the start of organ culture, ten explants per group were exposed for 6 hours to 0, 0.001, 1.0, and 10 µg of MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) per ml of fresh Waymouth's medium with no serum. Immediately following the last exposure, the explants were placed on the bottom of tissue culture dishes to facilitate outgrowths. The primary cultures from both MNNG exposed and control explants appeared morphologically similar during the first 3 months after carcinogen exposure. Figure 1 shows a typical 6-week-old primary culture from a control explant. These primary cultures consisted of epithelial

F-1

cells which were polygonal shaped, with distinct cell borders and contained occasional dome-shaped structures referred to as hemicysts. After approximately 120 to 140 days, small focal areas of high mitotic activity appeared in primaries from carcinogen exposed explants. One such "highly proliferating focus" is shown in Figure 2. As these areas expanded to the periphery of the culture, the cells became irregular-shaped and did not spread out like the controls, but rather remained tightly packed at the edge of the culture. These small irregular shaped cells which had a high nuclear to cytoplasmic ratio grew very rapidly covering the surface of the dish. After approximately 10^6 of these "morphologically altered" cells had accumulated, the cultures were dissociated in an attempt to establish cell lines. F-2

The numbers of explants yielding long-term primary cell cultures and cell line cultures is shown in Table 1. The number of explants in each group of 10 which produced one or more long-term primary cultures is shown in Column 2 of this table. Most of the explants, including the DMSO exposed controls, yielded primary cultures; however, as shown in the 3rd column, no cell lines could be established from primaries derived from unexposed explants. T-1

The average number of long-term primary cell cultures and cell line cultures produced per explant for the 10 explants in each group are shown in Table 2. Explants exposed to 1.0 or 10 µg MNNG/ml produced 2 to 3 times as many primary cultures per explant as controls, while exposure to 0.001 µg MNNG/ml produced about the same number of primaries as controls. T-2

A larger number of cell line cultures could be established with increasing exposure concentration of MNNG. Although significantly more cell lines could be established from explants exposed to 10 µg MNNG/ml than from those exposed to the lowest dose, the relative frequency with which cell lines could be established from primary cultures was approximately the same (i.e., the average number of cell lines per explant divided by the average number of primaries per explant). An example of one such cell line established from a primary derived from an explant exposed to 10 µg MNNG/ml is shown in Figure 3. F-3

The cell line cultures have been injected at about the 10th passage into the thighs of immunosuppressed isogeneic rats. Of these, 17 have produced palpable tumors that are presently 1 cm in diameter or larger. Eleven of the tumorigenic lines are derived from the explants exposed to 10 µg MNNG/ml, 4 from the explants exposed to 1 µg MNNG/ml, and 1 from those exposed to 0.001 µg MNNG/ml. Tumors produced by 8 of these cell lines, 6 from the 10 µg/ml exposure group, and 2 from the 1.0 µg MNNG/ml exposure group have been removed and histologically examined. Six of the tumors were squamous and 2 were adeno-squamous carcinomas which verifies the epithelial nature of the injected cells. Figure 4 is a histological section of one of these tumors showing its highly invasive properties. The other 9 tumors have not as yet been removed from the animals. We are now in the process of testing the oncogenicity of the non-tumorigenic cell lines at later passages.

These results show that neoplastic transformation of tracheal epithelium can be achieved <u>in vitro</u>. The current tumor data indicate that

more tumorigenic cell lines are derived from explants exposed to higher MNNG concentrations than from those exposed to lower concentrations.

Exposure of Tracheal Explants to TPA

The next step in establishing this system was to determine if the promoting agent TPA would stimulate DNA synthesis in tracheal cells in organ culture. The major variables studied were exposure modes and media conditions at various TPA concentrations. TPA exposure was started on day 9 of organ culture and ^{3}H-TdR incorporation was measured by autoradiography at various time points after exposure. Autoradiographic analysis of the various tissue layers of the explants showed that the major tissue to respond to TPA in terms of increased levels of DNA synthesis was the luminal epithelium. This occurred under all conditions tested. The number of labeled cells/mm of luminal epithelium was used as a measure of DNA synthesis.

Figure 5A summarizes the effects of repeated exposure to TPA (i.e., TPA was added every 3 days with each media change). In the organ cultures exposed to 0.25 µg TPA/ml a significant increase in the number of labeled cells/mm was only seen at day 6. However, DNA synthesis in explants exposed to 1.0 µg TPA/ml was increased 2.5 fold above the controls on days 3, 6, and 19. F-5

For the 24-hour exposure mode (Figure 5B), four different concentrations of TPA were added to the medium on day 0 (day 9 of culture) and removed 24 hours later. A greater than 4-fold increase in DNA synthesis

over control values occurred at 24 hours with the two lowest TPA concentrations (0.016 and 0.063 µg/ml). With the higher TPA concentrations of 0.25 and 1.0 µg/ml, a marked increase in labeling was not seen until days 3 and 6 respectively.

The data obtained with a single 1-hour exposure to TPA are summarized in Figure 5C. Exposure to 1.0 µg TPA/ml for only one hour increases the number of labeled cells/mm 3 fold at 1 day. In contrast, there is no stimulation after 24 hours of exposure to the same concentration of TPA (Fig. 5B). A single 1-hour exposure to the 3 lower doses did not stimulate DNA synthesis until 6 days.

The next series of experiments was conducted to determine the effect of medium composition on the response elicited by TPA and to provide a basis for choosing a medium for future promotion studies. Marchok (unpublished results) found that medium containing low levels of insulin and hydrocortisone help to maintain the mucociliary epithelium of the tracheal explant in organ culture in a highly differentiated state. Figure 6A F-6 summarizes the studies on the effect of insulin and hydrocortisone on TPA-induced DNA synthesis. The "repeated" mode of exposure to TPA was used, that is, TPA was added with fresh media every 3rd day at the time of media changing. Tritiated thymidine incorporation by the epithelium decreases at one day when explants are cultured in medium containing hormonal supplements (Fig. 6A), while no decrease is observed using media without these supplements (5A). Although ^{3}H-TdR incorporation by the epithelium is greater in the hormone supplemented medium, the percent stimulation above control levels after TPA exposure is approximately the same as without these supplements.

The effect of increasing the serum level in the medium from 2% to 5% are shown in Figure 6B. The lack of stimulation of DNA synthesis at the higher serum level is surprising since only a 3% difference exists. The higher serum concentration may be binding or inactivating a significant amount of TPA.

These results show that tracheal explants in vitro respond to TPA exposure by a marked increase in DNA synthesis. This stimulation was observed at low serum levels and the highest stimulation was obtained with short exposures to TPA. The optimal experimental conditions were implemented in the design of the following initiation-promotion experiment.

Initiation Promotion Study

Tracheal organ cultures were exposed on days 3 and 6 to MNNG as in the first study. Beginning 3 days after carcinogen exposure, the organ cultures were also exposed to TPA for 1 hour every 6 days for 3 or 6 weeks. At the end of this period, the explants were placed in outgrowth culture. The major objectives of this experiment are to determine if there is either an increase in the frequency of explants which will yield transformed cell lines and/or a decrease in the average time required to produce transformation by exposure to TPA. The preliminary results are given in Table 3. These data show the number of explants T-3 in each group of 10 which gave rise to primaries currently having "morphologically altered" (MA) cells (i.e., irregularly-shaped cells which maintain a compact appearance at the growing edge of the culture and grow very rapidly across the surface of the dish). These MA cells

are similar to those described earlier in the first study. Since the results obtained for explants treated for 3 and 6 weeks respectively with TPA are very similar at this point, they are grouped together. In the low MNNG dose groups, TPA clearly increased the number of explants producing MA cells at 6 months. With the high MNNG concentration, this effect was less noticeable. TPA alone, without prior MNNG exposure, also produced some explants yielding MA cells.

Not shown in Table 3 are earlier results obtained at 3 months after MNNG exposure. At that time only 2 explants of the 10 which were exposed to the high MNNG concentration alone had produced primary cultures with MA cells, while 5 explants in the group exposed to 1.0 µg MNNG-1.0 µg TPA/ml produced primaries with such cells. Thus TPA seems to accelerate the appearance of MA cells. No "MA" cells have been seen in the DMSO-exposed control cultures. We are presently in the process of establishing cell lines from the "MA" primary cultures. Since cell lines could be established from nearly all cultures having morphologically altered cells in the experiment with MNNG only, we anticipate establishing cell lines from all the primaries having "MA" cells in this promotion study. Presently two cell lines have been established from tissue exposed to TPA alone. The data, although preliminary, suggest that TPA enhances the MNNG effect, particularly at the lower MNNG concentration.

DISCUSSION

We show, using an organ culture-cell culture system, that rat tracheal epithelium can be transformed *in vitro* by exposure to MNNG. Preceeding neoplastic transformation, several carcinogen-dose dependent changes were observed in the growth behavior of the epithelial cells. First of all, the number of primary epithelial cell cultures that can be obtained per explant increases as MNNG exposure concentration increases. In these primary cultures an early change in growth rate was noticed which manifested itself by the appearance of foci composed of rapidly proliferating cells. These foci, which did not appear in control cultures, preceeded the appearance of "morphologically altered" cells. Only primary cultures containing these morphologically altered cells could be subcultured repeatedly into cell lines. Secondly, a greater portion of these cell line cultures could be established per explant with increasing MNNG exposure concentration. Thirdly, this dose dependent effect is reflected in the tumor yield. More cell lines are tumorgenic at higher, compared to lower, MNNG exposure concentrations. This phase of the study is still in progress.

In the second series of experiments, it is shown that tracheal organ cultures, exposed to TPA *in vitro*, respond by a marked increase in DNA synthesis. The response is similar to that described for mouse skin (1,2,9,12,13). The characteristics of this response are dependent on TPA concentration, exposure mode (i.e., repeated versus single exposure and exposure duration), and media composition. It is found that TPA concentrations of 0.25 or 1.0 μg/ml provide the maximum sustained

stimulatory effect of DNA synthesis in luminal epithelium, although a significant short-term stimulation is obtained with TPA concentrations as low as 0.016 µg/ml. Our results also show that the shorter single exposures of 1 hr or 24 hrs, stimulate ^{3}H-TdR incorporation sooner and at higher levels than continuous exposure. From these studies, it was decided to use a one-hour exposure to 1.0 µg TPA per ml media every 6 days in an initiation-promotion experiment which is now in progress.

In the third set of experiments, the initiation-promotion study, organ cultures are exposed first to MNNG, followed by TPA exposure, then epithelial primary cultures are obtained by the outgrowth method. To date, exposure to both agents results in an enhancement in the number of explants producing morphologically altered cells compared to exposure to either agent alone. Currently we are establishing cell lines from these morphologically altered cells and will determine tumorigenicity by injection into isogenic recipients.

Recent studies by Lasne et al. (3), Mondal et al. (7), and Mondal and Heidelberger (8) strongly suggest that two-stage carcinogenesis can be reproduced in vitro in fibroblast cell lines. The organ culture-cell culture model system described here utilizes epithelial tissue and has several other desirable features: (a) the tissues during exposure closely resemble the tracheal mucosa in vivo; (b) selection processes appear to be minimal in the establishment of primary cultures; (c) primary cultures, exposed or unexposed, can be maintained for many months which may be necessary for the transformation of many of the initiated cells;

(d) spontaneous transformation has not, as yet, been observed, while a high percentage of exposed tissues are transformed.

Perhaps the most important feature of this experimental system is the early appearance of rapidly-proliferating, morphologically-altered cells. We have demonstrated that this morphological change preceeds the ability to subculture and, therefore, is a marker indicating an increased in vitro growth capacity of the cells. The development of a seemingly "unlimited" growth capacity appears to be an essential element, if not a prerequisite, for the expression of neoplastic transformation by the epithelial cells studied here. However, this change does not always lead to neoplastic transformation. Marchok et al. (5) have shown that cell lines derived from tracheal epithelium, exposed to low levels of carcinogen in vivo, do not show evidence of transformation after more than 30 passages, representing over 400 days in culture. In addition, we find that exposure of tracheal explants to TPA alone in some cases leads to the establishment of cell lines. Further studies will be required to clarify the relationship between a change in in vitro growth capacity and neoplastic transformation. Although promotion has not, at this point, been demonstrated, we are encouraged by the initial results and hope that this system will allow the detailed study of multistage carcinogenesis in vitro. Such studies should answer many important questions concerning the mechanisms of respiratory tract cancer induction.

SUMMARY

An organ culture-cell culture system was used to demonstrate carcinogen dose-dependent transformation of tracheal epithelial cells *in vitro*. Tracheal explants were exposed to MNNG in organ culture. Outgrowths from these explants provided epithelial cell cultures. The numbers of long term epithelial cell cultures and cell lines that were established per explant increased as MNNG exposure concentration increased. At the present time, more cell lines derived from explants exposed to the highest MNNG concentration have produced palpable tumors than cell lines derived from explants exposed to lower MNNG concentrations. No cell lines were established from primaries derived from control explants.

TPA stimulates DNA synthesis in tracheal epithelium in organ culture in a manner similar to that described for mouse skin. Short exposures to TPA not only stimulated DNA synthesis earlier, but the stimulation was greater than that obtained with "continuous" exposure.

At the present time, exposure of tracheal organ cultures to MNNG followed by TPA has resulted in an enhanced production of "morphologically altered" cells in primary epithelial cell cultures, than exposure to either agent alone.

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FIGURE LEGENDS

Fig. 1. Six-week-old primary epithelial cell culture from a control rat tracheal explant. These small, compact, polygonal cells with occasional hemicysts (arrows) were also characteristic of early primaries from MNNG-exposed explants. Phase contrast, X100.

Fig. 2. Mitotically-active focus in a primary culture from an explant which was exposed to 1 µg/ml of MNNG. Cells in the focus appear as a multi-layered, smooth sheet of pale cells with many mitotic figures (arrows). Foci such as this appeared in primary cultures at approximately 120 days after carcinogen exposure. Phase contrast, X100.

Fig. 3. Established epithelial cell line of irregular-shaped, rapidly growing cells from an explant exposed to 10 µg/ml of MNNG, phase contrast, X100.

Fig. 4. Highly invasive squamous cell carcinoma observed following injection of 10^6 cells from an established epithelial cell line, derived from an explant exposed to 1 µg/ml of MNNG. H & E, X248.

Fig. 5. Effects of various TPA exposure modes and doses on the incorporation of [^{3}H] thymidine into luminal epithelial cells of tracheal organ cultures. The culture media used was Waymouth's + 2% FBS. A. Repeated exposure (TPA was added with fresh media every third day at the time of media changing). B. Single 24-hour exposure beginning at day 0.

C. Single 1-hour exposure. Three explants, four histological sections each, were used for each point. Symbols: ○, 0 µg/ml; ▣, 0.016 µg/ml; ◈, 0.063 µg/ml; ▲, 0.25 µg/ml; ●, 1.0 µg/ml TPA.

Fig. 6. Effects of media composition on the incorporation of [^{3}H]-thymidine into luminal epithelial cells after repeated mode exposure to TPA. A. Waymouth's + 0.1 µg/ml insulin + 0.1 µg/ml hydrocortisone + 2% FBS. B. Waymouth's + 0.1 µg/ml insulin + 0.1 µg/ml hydrocortisone + 5% FBS. Three explants, four sections each, were used for each point. Symbols: ○, 0 µg/ml; ▲, 0.25 µg/ml; and ●, 1.0 µg/ml TPA.

TABLE 1: Number of tracheal explants producing primary epithelial cell cultures and cell lines from tracheal explants exposed to MNNG *in vitro*.

MNNG (μg/ml)[a]	No. explants yielding primary cultures[b]	No. explants yielding cell lines[c]
0	6	0
0.001	9	7
1.0	10	8
10.0	10	10

[a]The MNNG was initially dissolved in dimethyl sulfoxide (DMSO) and the final media concentration of this solvent was 0.5%. The control group was exposed to 0.5% DMSO only.

[b]Ten explants per group were exposed. Explants were replanted 8-10 times to obtain multiple outgrowths. These outgrowths were designated primary cultures if they survived more than 3 weeks after the removal of the explant.

[c]Cell cultures were designated cell lines after the 5th passage.

Data from Steele, *et al.*, (10)

TABLE 2: Number of primary cell cultures and cell lines produced per tracheal explant after exposure to MNNG *in vitro*.

MNNG (µg/ml)	Mean number of primary cultures per explant (± S.E.)[a]	Mean number of cell lines per explant (±S.E.)[a]
0	1.3 ± 0.4	0
0.001	1.5 ± 0.3	0.8 ± 0.2
1.0	3.3 ± 0.8	1.3 ± 0.3
10.0	4.6 ± 0.6[b]	2.0 ± 0.3[b]

[a]Of a possible 10 primaries or 10 cell lines per explant due to the replanting method used.

[b]These values are significantly different from the control and the lowest MNNG dose at the $p = 0.05$ level.

Data from Steele, *et al.*, (10).

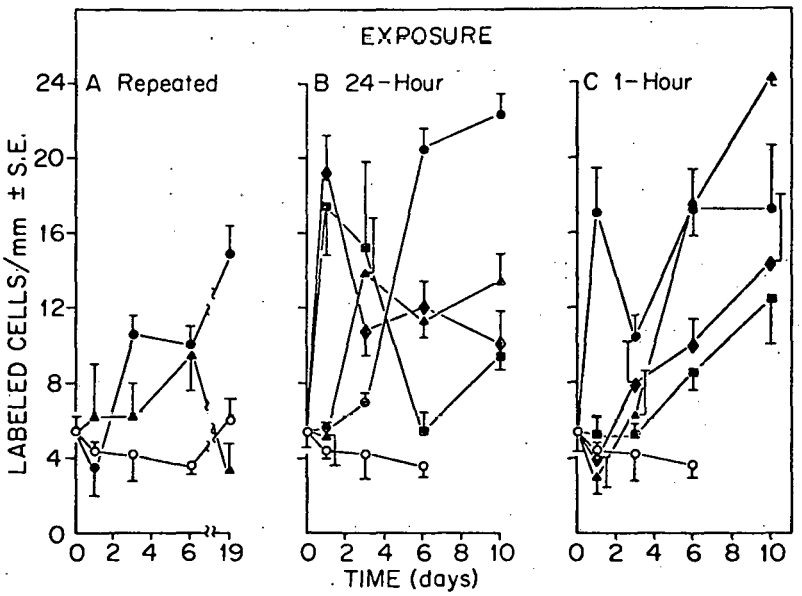
TABLE 3: Number of explants producing morphologically altered (MA) cells following exposure to MNNG and/or TPA. Preliminary results six months after carcinogen exposure.

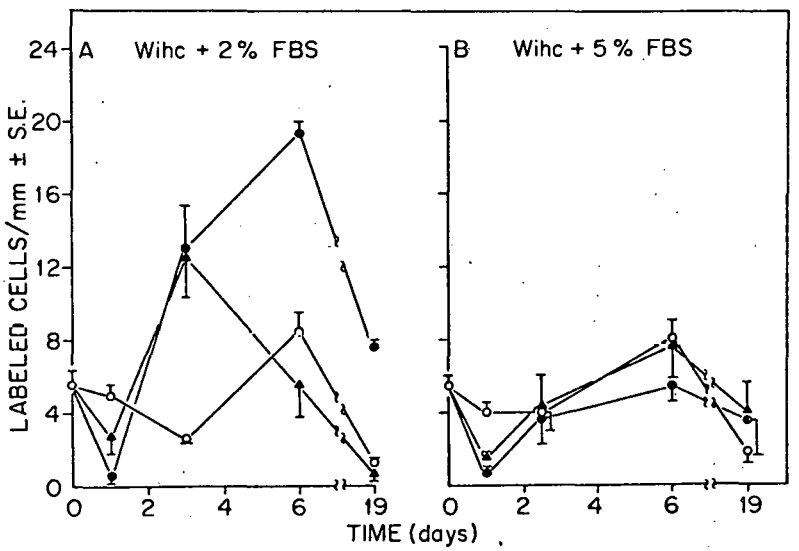
	TPA (μg/ml)[a]		
	0	0.01	1.0
MNNG (μg/ml)	MA	MA	MA
0	0[b]	3	3
0.0001	4	8	10
1.0	6	8	8

[a] TPA exposure was for 1 hour every 6 days for 3 or 6 weeks beginning at day 9.

[b] Each exposure group contained 10 explants.

3
1
4
2


EXPOSURE
A Repeated
B 24-Hour
C 1-Hour
LABELED CELLS/mm ± S.E.
0
4
8
12
16
20
24
0 2 4 6 19
0 2 4 6 8 10
0 2 4 6 8 10
TIME (days)


A Wihc + 2% FBS
B Wihc + 5% FBS
LABELED CELLS/mm ± S.E.
24
20
16
12
8
4
0
0
2
4
6
19
TIME (days)