In Vitro Alterations of Tracheal Epithelium of Hamsters by Carcinogens in Organ Culture

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HE, M., HUN, S., ZHANG, L. and LIAN, C. In Vitro Alterations of Tracheal Epithelium of Hamsters by Carcinogens in Organ Culture. Tohoku J. exp. Med., 1987, 152 (1), 87-100 — In vitro organ culture of the hamster's trachea was improved and applied to a carcinogenesis research. The rotary culture enabled explants of tracheal epithelium to survive more than 8 weeks. The study was composed of 2 kinds of culture; untreated and treated with carcinogens. In the untreated culture, Eagle MEM medium had the same culture effect as RPMI 1640 medium. With prolongation of culture time (particularly longer than 5 weeks), irreversible degenerative changes appeared in epithelial cells. Culture for 4 weeks was usually thought to be appropriate for experimental research. In the treated culture, the effect of benzo-(a) pyrene (B(a)P) and B(a)P+cigarette smoking condensate-neutral fraction (CSC-NF) on tracheal epithelium was investigated with light and electron microscopies (TEM and SEM) and autoradiography. Atypical hyperplasia with or without lesions suggesting carcinoma in situ was induced by B(a)P+CSC-NF more evidently and frequently than by B(a)P alone. The present findings corroborated the cocarcinogenetic effect of CSC-NF.

trachea; hamster; rotary culture; CSC-NF; cocarcinogenesis

Organ culture is different from the cell culture basically in keeping the tissue structure throughout the experiment and particularly applied to the research on tissue transformation in vitro. This method has been widely used in the study of carcinogenesis due to an advantage of its short course for observing the direct effect of carcinogens.

The organ culture of respiratory tissues was introduced by Lasnitzki into the carcinogenesis research in vitro using the human fetal lung (Lasnitzki 1958, 1968). Afterward, several reports of tracheal organ culture have been made on rat, hamster and man (Palekar et al. 1968; Barrett et al. 1976; Carson et al. 1980; Trump et al. 1980; Chopra and Cooney 1983; Mass and Kaufman 1983; Woodworth et al. 1983).

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The present paper deals with the organ culture of hamster's trachea in various conditions. Changes of tracheal epithelium with varying the cultural time (1-8 weeks) and the nutritional medium will be demonstrated. The aim of this study is to reveal the cocarcinogenetic activity of CSC-NF (cigarette smoking condensate-neutral fraction) in vitro.

**MATERIALS AND METHODS**

*Preparation of tracheal rings.* Female golden hamsters weighing 120 g, total 150 in number, were anesthetized by an intraperitoneal injection of pentobarbital. The trachea was removed under the sterile condition, washed 3 to 4 times in Hanks solution, and cut in 10 pieces (tracheal ring), 1.0 mm thick. Several (or 4 to 6) pieces of randomly picked rings were put in the Kelle flask (20 ml).

*Culture condition.* The medium RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo) or Eagle MEM (Nissui Pharmaceutical Co., Ltd., Tokyo) containing 100 U/ml of penicillin, 0.1 mg/ml of streptomycin, 0.1 mg/ml of hydrocortisone, 1 mg/ml of insulin and 0.3 mg/ml of glutamine. Cultures were incubated in the rotary apparatus with a humidified atmosphere of 5% CO₂ at 36.5-37°C. The rotary speed of the disc of apparatus was 7.5 time/hr. The media were changed every day as long as 8 weeks.

*Carcinogens.* For carcinogens added to the culture medium, benzo(a)pyrene (B(a)P) and cigarette smoking condensate-neutral fraction (CSC-NF; Koch-Light Laboratories Ltd., Colnbrook Bucks, England) were used. CSC-NF is prepared from cigarette's tar after artificial smoking (the cigarettes are made by Shenyang Cigarette Co., Shenyang, P.R. China) and extracted under the assistance of Department of Chemistry at China Medical University.

*Experimental groups.* The whole experiments were composed of 2 groups of untreated and treated culture and each was subdivided as follows:

A. **Untreated culture**
   - Group 1. RPMI 1640
   - Group 2. RPMI 1640 with 5% calf serum
   - Group 3. Eagle MEM
   - Group 4. Eagle MEM with 5% calf serum

B. **Treated culture**
   - Group 1. (untreated group). RPMI 1640
   - Group 2. (solvent control group). RPMI 1640 containing 0.2% acetone
   - Group 3. (B(a)P group). B(a)P (6 μg/ml + RPMI 1640) dissolved in acetone (0.2% final)
   - Group 4. (CSC-NF group). CSC-NF (100 μg / ml + RPMI 1640) dissolved in acetone (0.2% final)
   - Group 5. (Mixed group). A mixture of B(a)P and CSC-NF. (The concentration of B(a)P and CSC-NF was the same as group 2 and group 3, respectively.)

*Light microscopic observation.* Ciliate movement of the trachea epithelium was observed under an inverted (or phase contrast) microscope during the culture process. Four pieces of the cultured trachea were taken from each group per week and prepared for further investigations. Histologic sections were made and stained with hematoxylin and eosin (HE). For control, fresh specimens of the hamster's trachea were examined.

*Electron microscopic observations.* The specimens fixed in 2.5% glutaraldehyde for 4 hr were washed in phosphate buffer and postfixed in OsO₄ for 2 hr. The tissues were dehydrated in a graded ethanol series and embedded in Epon 812. Thin sections stained with uranyl acetate were observed with a TEM Hitachi H-600. Scanning electron-microscopic observations were performed by the routine procedure using a SEM Hitachi S-450.

*Autoradiographic studies.* Specimens from each group at different culture time were
put into the medium 1640 or Eagle MEM with 5 μCi/ml ³HtdR and incubated for 1.5 hr at 36.5°C. Then the specimens were washed 3 times with Hanks solution and fixed in 10% formalin. Thin sections were coated with NTB-2 photographic emulsion and exposed in a dark room for 2 weeks for autoradiograms.

**RESULTS**

*Untreated culture*

Alterations of pseudostratified ciliated (PC) columnar epithelium with dis-

![Image](image.png)

Fig. 1. Tracheal epithelium remains to be of pseudostratified ciliated columnar structure at 2 weeks culture, RPMI 1640 + 5% calf serum.

<table>
<thead>
<tr>
<th>Time (week)</th>
<th>G1 RPMI 1640</th>
<th>G2 RPMI 1640 + calf serum</th>
<th>G3 Eagle MEM</th>
<th>G4 Eagle MEM + calf serum</th>
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Direction of changes: PC → SC → SS

Fig. 2. Graphic demonstration of epithelial lesions in the hamster's trachea with the lapse of organ culture. PC, pseudostratified ciliated; SC, simple columnar; SS, simple squamous.
tinct cilia of the trachea were induced with the lapse of culture time similarly in
the 4 untreated groups. In 1 week of culture, some cultured epithelia changed to
a simple columnar (SC) type and only a few to a simple squamous (SS) type, while
others maintained the primary structure (Fig. 1). In 3–4 weeks, the epithelium
exhibited mostly SC and partly SS type. In 5–6 weeks, the epithelium was of SS

Fig. 3. Severe atypical hyperplasia involving the whole layer of tracheal epithelium (suggestive of carcinoma in situ) treated by B(a)P at 8 weeks culture, RPMI 1640 + acetone + 5% calf serum. (HE × 400)

Fig. 4. Severe atypical hyperplasia involving the whole layer of tracheal epithelium (suggestive of carcinoma in situ) treated by B(a)P + CSC-NF at 6 weeks culture, RPMI 1640 + acetone + 5% calf serum. (HE × 100)
type in most cases and of SC type in a few. In 7–8 weeks, a few were still characterized by the SC type. In summary, transformations of tracheal epithelium with the lapse of culture time proceeded along a spectrum of PC–SC–SS (Fig. 2), irrespective of the type of culture medium; Eagle MEM or RPMI 1640, or the presence or absence of calf serum.

Treated culture

*B(a)P Group.* In 4 weeks of culture, local proliferation of epithelial cells regular in size and arrangement formed a small nodular protrusion to the tracheal lumen. In 6–8 weeks, epithelial proliferation was more evident than that in 4 weeks and composed of atypical cells irregular in size and arrangement compared with those in 4 weeks. The nucleus heavily stained was large and occasionally associated with a mitotic figure. Atypical hyperplasia was confirmed in 5 cases and seemingly carcinoma in situ in 1 case (Fig. 3).

*B(a)P + CSC-NF Groups.* Epithelial hyperplasia appeared early and became much obvious in 4 weeks of culture manifesting 8–10 layers of proliferation. The proliferating cells were irregular both in size and arrangement. The nucleus was generally large and heavily stained associated with prominent mitotic figures. In three cases marked proliferation of atypical cells involving the whole layers of epithelium was evidently suggestive of carcinoma in situ (Fig. 4).

*CSC-NF Groups.* Only some slight epithelial proliferation was observed in 4 weeks and 5 cases of squamous metaplasia in 6 weeks.

*Untreated and solvent control Groups.* Epithelial alterations in these groups were fundamentally the same as the above findings in the untreated culture groups. Furthermore, only a few cultured epithelia showed slight hyperplasia in 6 weeks of culture.

Table 1 shows the epithelial findings, examined with a light microscope, in

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<th>Table 1. Findings of tracheal epithelium cultured for 6 weeks</th>
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<td>Unchanged epithelium</td>
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<td>Multilayer hyperplasia</td>
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<td>Squamous metaplasia</td>
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<td>Atypical hyperplasia</td>
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<td>Carcinoma in situ</td>
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* Number of tissues showing lesions.
the untreated and treated groups cultured for 6 weeks.

**Electron microscopic findings**

*Transmission electron microscopy (TEM)*

TEM observations revealed that tracheal epithelium at-zero time was in most part composed of ciliated cells, mucous cells and brush cells with some basal cells beneath them. The ciliated cells were clear cells of tall columnar shape with lower electron density. They had oval nucleus and clear cytoplasm with a number of mitochondria and a few rough endoplasmic reticulum. The mucous cells were columnar in shape with some microvilli on the luminal surface. They contained some intraplasmic mucous vesicles of high electron density. The brush cells were located between ciliated cells and/or mucous cells. The brush cells were cubic or columnar in shape, containing some rER and ribosomes in the cytoplasm, and covered with discrete microvilli on the surface. At zero time and early stage of culture, the abovementioned cells adjacent to the superficial portion showed tight intercellular junctions.

In all 4 groups of untreated culture, most epithelial cells maintained their normal features at early culture time (Fig. 5), but cilia of some explants were about to be exfoliated. Flat nucleus, dilated cisternae of rER as well as a large amount of ribosomes and lysosomes could be seen (Fig. 6).

![Image of a part of ciliated cell with oval nucleus clear cytoplasm and a number of mitochondria. Neighboring brush cells are of cubic shape and covered with discrete microvilli. Untreated group. At 1 week culture, RPMI 1640+5% calf serum. (TEM ×4,000)](image-url)
In 5 weeks of culture, the epithelium appeared to form monolayer and, meanwhile, a number of degenerated lysosomes, autophagic vacuoles and ribosomes were observed in the cytoplasm.

In 7-8 weeks, most of the tracheal epithelia changed to the single cell layer while the remnant still kept their primary structure (Fig. 7). The changed epithelium had ciliated cells without cilia and with long oval nuclei lying parallel to the surface (Fig. 8). Some enlarged lysosomes and autophagic vacuoles were observed in the cytoplasm, suggesting cytoplasmic degeneration.

Epithelial hyperplasia with disarranged multilayer cells was observed in 3 treated groups of CSC-NF, B(a)P and CSC-NF+B(a)P (Fig. 9).

In the CSC-NF group after 4 weeks culture the cilli of epithelial cells were lost and scant organelles in cytoplasms could be seen. In B(a)P and B(a)P + CSC-NF groups at the same culture time a lot of microfilaments were observed in
Fig. 7. Tracheal epithelium is still of primary structure and contains ciliated cells in the right 2 cells. Left 3 mucus cells are of integrity at 7 weeks culture, Eagle MEM. (TEM ×4,000)

Fig. 8. The ciliated cell with a few cilia and long oval nucleus lying parallel to the surface of cell at 8 weeks culture of the untreated group, Eagle MEM. (TEM ×5,000)
some superficial cytoplasms, and the interdigitate junctions and desmosomes increased among adjacent cells.

Epithelial hyperplasia with disarranged multilayer cells were most obvious. The nuclei were enlarged and N/C ratio was distorted. Block-shaped chromatin beneath the nuclear membrane as well as the foldings and depressing or deep incisions of nucleus could also be seen. These changes in both B(a)P and B(a)\textsuperscript{P}+CSC-NF groups corresponded with those of severe atypical hyperplasia and carcinoma in situ were observed under LM.

*Scanning electron microscopy (SEM)*

In 4 weeks of culture, numerous ciliated cells with dense cilia and some nonciliated cells oval in shape were observed in the untreated culture groups. Nonciliated cells were covered with discrete microvilli on the surface (Fig. 10). After 5 weeks of culture, the field of nonciliated cells was generally enlarged. The superficial cilia of ciliated cells became shorter and scarce, and further
exfoliated somewhere.

In 4 weeks of the CSC-NF group, there were broad areas of enlarged nonciliated cells, which had rough surface and were covered with microvilli. Cilia were missed on the surface of most ciliated cells (Fig. 11).

In the B(a)P and B(a)P + CSC-NF groups, epithelial proliferation of various grade was prominent. Some nonciliated cells were arranged in a hill-form which protruded into the tracheal lumen or possessed rough, wrinkled surfaces with raised cell margins (Fig. 12). These appearances were consistent with atypical hyperplasia of epithelial cells under a light microscopy.

Radioautography

In any of 4 untreated and 5 treated groups at every culture time, $^3$HTdR was incorporated in nulei of superficial and basal cells of tracheal epithelium (Figs. 13 and 14).

Discussion

Tracheal organ culture and its application

Organ culture has been used for researches on the detection of carcinogenetic factors and on carcinogenesis and infiltrative growth of a tumor. This is of great advantage to the experimental study in its shortness in time and easiness in controlling factors.
Fig. 11. There were no cilia on ciliated cells at 4 weeks culture of the CSC-NF group, RPMI 1640 + 5% calf serum + acetone. (SEM × 2,500)

Fig. 12. Some nonciliated cell represented a hillform and are protruded into the tracheal lumen or possessed of rough, wrinkled surfaces with raised cell margin at 4 weeks culture of the B(a)P group, RPMI 1640 + 5% calf serum + acetone. (SEM × 2,000)
We have confirmed that epithelium of the cultured hamster’s trachea changes its morphological features with the difference of time and condition of the culture. The survival of the cultured trachea in RPMI 1640 and Eagle MEM medium might be maintained in 8 weeks. Previous reports (Mossman and Craighead 1975; Mossman et al. 1978) on trachea organ culture suggested that the basic medium such as Eagle MEM maintained the primary differentiated structure in

Fig. 13. ³HTdR incorporated into nuclei of superficial and basal cells of epithelium at 2 weeks culture of the untreated group, RPMI 1640+5% calf serum. (Radioautograph ×200)

Fig. 14. Same changes as Fig. 14 at 4 weeks culture, RPMI 1640+5% calf serum. (Radioautograph ×200)
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culture longer than CMRL-1066 and that the latter induced the epithelium to hyperplasia and squamous metaplasia. The result that \(^{3}\)HTdR was significantly incorporated into tracheal epithelium of hamster by autoradiographic studies gave evidence of some constructive metabolism in the cultured epithelial cells (Kaufman et al. 1973; Harris et al. 1976).

Recent progress of cultivation using rotary techniques has been successful for the prolongation of culture time, but a series of epithelial alterations in vitro could not be avoided. These alterations such as exfoliation of cilia of epithelial cells resulting in squamous metaplasia, changes of organelles and other cytoplasmic degenerative changes were observed at the level of both histology and ultrastructure.

Our results indicated that the integrity of tracheal epithelium is kept for 4 weeks and this is adequate enough to carry out various treatments for research.

**In vitro precancerous and carcinomatous changes by carcinogens**

Lasnitzki (1968) studied the in vitro effect of B(a)P on bronchiolar epithelium by culture of the human fetal lung and found the precancerous changes in 4 weeks of culture. In our study epithelial hyperplasia was evident in the B(a)P group and B(a)P+CSC-NF group in 6–7 weeks and 3–4 weeks, respectively. These hyperplastic cells were irregular in size, shape and arrangement. The nuclei were enlarged and deeply stained. These findings were generally regarded as severely atypical hyperplasia that belongs to precancerous changes. The ultrastructural findings observed under TEM and SEM were consistent with light microscopic observations. Furthermore, in 6 weeks of culture, remarkable changes suggestive of carcinoma in situ were observed in 3 cases in the B(a)P+CSC-NF group and one in the B(a)P group. Therefore, the trachea organ culture might be a model of in vitro carcinogenesis or inducing preneoplastic lesions and useful for the detection of tumorigenic agents.

**Co-carcinogenetic effect of CSC-NF**

In recent years, the effect of CSC-NF on promoting carcinogenesis and co-carcinogenetic activity has been actively studied (Van Duuren et al. 1971, 1973; Bock and Park 1972; Bouffant et al. 1980; Steels et al. 1983). In the present study preneoplastic and in situ-carcinomatous changes of tracheal epithelium were more evident in the mixed group than the B(a)P group.

At 6 weeks of culture, atypical hyperplasia and carcinoma in situ in the mixed group were higher in incidence than in the B(a)P group. The difference between these two groups was significant. The lesions in the mixed group were not only frequent and intensively severe but also very early. Therefore, the authors should consider that the above-mentioned results might be induced not merely by B(a)P but most probably by the association with a co-carcinogenetic activity of CSC-NF.
Acknowledgments

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References