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Neoplastic potential of rat tracheal epithelial cell lines induced by 1-nitropyrene and dibenzo(*a*,*i*)pyrene [1]

M.-X. Ensell [a,b], A. Hubbs [b], G. Zhou [b], L. Battelli [b], J. Nath [a], T. Ong [a,b,*]

[a] *Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, MS 3014, 1095 Willowdale Road, Morgantown, WV 26505, USA*

[b] *Genetics and Developmental Biology Program, College of Agriculture and Forestry, West Virginia University, Morgantown, WV, USA*

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Abstract

Our previous study showed that both 1-nitropyrene (1-NP) and dibenzo(*a*,*i*)pyrene (DBP) induced enhanced growth variants (EGVs) in primary cultures of rat tracheal epithelial (RTE) cells exposed in vivo. Cell lines were established from some of the EGVs. Further studies, using anchorage-independent growth in soft agar and tumorigenicity in athymic nude mice, were performed to determine the neoplastic potential of EGVs induced by 1-NP and DBP. Results show that three of five from DBP- and five of five from 1-NP-induced cell lines displayed anchorage-independent growth. The colony forming efficiency (CFE) from DBP-induced cell lines was 0.067‰ and CFE from 1-NP-induced cell lines was 0.151‰. There is a significant difference between the two CFEs ($\mu = 12.08$, $P < 0.01$). Two of five DBP- and five of five 1-NP-induced cell lines produced squamous cell carcinomas (SCC) in nude mice. The rate of tumorigenicity counted by injected sites was 20% (6/30) for DBP-induced cell lines and 57% (17/30) for 1-NP-induced cell lines. There is a significant difference between the results of tumorigenicity from the cell lines induced by the two different compounds ($\chi^2 = 8.53$, $P < 0.01$). Neither of the two cell lines from spontaneously developed foci grew in soft agar or produced SCC in nude mice. It seems that the neoplastic potential of transformed RTE cells induced by 1-NP was higher than that of DBP.

Keywords: 1-Nitropyrene; Dibenzo(*a*,*i*)pyrene; Rat tracheal epithelial cell lines; Neoplastic potential; Soft agar; Nude mice

1. Introduction

Lung cancer has been increasing in incidence and is a predominant cause of cancer-related deaths in the United States [1]. It is estimated that occupational exposure causes about 10,000–12,000 cases of lung cancer annually in the US alone [2]. Understanding the mechanism of lung cancer has become an important issue in cancer research and prevention.

* Corresponding author. Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, MS 3014, 1095 Willowdale Road, Morgantown, WV 26505, USA. Tel.: +1-304-285-5760; fax: +1-304-285-6194; E-mail: too2@cdc.gov

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The rat tracheal epithelial (RTE) cell transformation system is a useful assay to detect potential pulmonary carcinogens [3,4]. In this assay system, enhanced growth variants (EGVs), sometimes referred to as transformed foci, which are induced by carcinogens, are derived from normal primary RTE cells. Cell lines can be established from EGVs and the neoplastic potential of the cells from these cell lines can be analyzed by anchorage-independent growth in soft agar and tumorigenicity in athymic mice. The entire process resembles the multiple stages of carcinogenesis in vivo.

1-Nitropyrene (1-NP), the predominant nitropolycyclic hydrocarbon found in diesel exhaust, is a ubiquitous environmental pollutant. Experiments in mammalian cells show that 1-NP formed DNA adducts with both in vivo [5] and in vitro [6] exposures. It has been reported that the addition of an exogenous metabolic system (rat liver S9) was essential for 1-NP to induce chromosomal aberrations (CAs) [7] and to increase sister chromatid exchanges (SCEs) [8] in mammalian cells in vitro. 1-NP and its derivatives also induced the transformation of cultured normal human skin fibroblasts to a stage of anchorage-independent growth and cellular invasiveness [9]. Most carcinogenesis studies have shown that 1-NP induces tumors in some species of rodents [10–14]. However, Tokiwa et al. [15] found that 1-NP is not carcinogenic in BALB/c mice. In vitro morphological transformation assays have shown that 1-NP induces cell transformation in BALB/c 3T3 cells [16] and Syrian hamster embryo cells [17], but not in RTE cells [18].

Dibenzo(a,i)pyrene (DBP) is one of the by-products found in many industrial wastes and emissions, including diesel exhaust. By intratracheal instillation in rats, DBP induced DNA adducts in lung tissue [19] and micronuclei in bone marrow cells and spleen erythrocytes [20]. Applied on mouse skin, DBP induced formation of DNA adducts in skin and lung cells [21] and skin tumors were induced following application of tetradecanoylphorbol acetate to the initiated skin [22].

In our previous study, we found that both 1-NP and DBP induced dose-related increases in frequencies of EGVs among RTE cells exposed in vivo to these agents (Table 1). The increases, with the exception of two lower doses of 1-NP, are statistically significant. The increase at the highest dose tested was about nine-fold that of the control animals [23]. This study compared the neoplastic potential of RTE cell lines established from transformed foci occurring spontaneously and those induced by 1-NP and DBP.

Table 1
Transformation efficiency of RTE cells exposed in vivo to DBP and 1-NP. Note: The data in this table is cited from Ref. [23]

Compound[a]	Dose (mg/kb bw)	TFE (%)[b]
DBP	3.75	1.23**
	7.50	1.97**
	15.00	3.26**
	30.00	4.35**
1-NP	7.50	0.65
	15.00	0.84
	30.00	2.26**
	60.00	4.66**
DMSO (solvent control)	3.00 (ml)	0.50
CSC (positive control)	3.00 (cigarettes)	1.70**
	6.00 (cigarettes)	1.50**
	12.00 (cigarettes)	5.48**

[a] DBP, dibenzo[a,i]pyrene; 1-NP, 1-nitropyrene; DMSO, dimethylsulfoxide; CSC, cigarette smoke condensate.
[b] Transformation efficiency (%) = (total enhanced growth variants/total cell colonies) × 100.
** $P < 0.01$.

2. Materials and methods

2.1. Cells, media and cell preparation

Viral antigen negative Sprague–Dawley (SD) male rats (200–220 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Rats were instilled with 1-NP at concentrations of 30 and 60 mg/kg bw or DBP at 15 and 30 mg/kg bw once every 3 days for a period of 9 days. Isolation and culture of the RTE cells were performed according to the method recommended by Thomassen et al. [4]. Tracheae were removed 24 h after last exposure and rinsed with Nutrient Mixture Ham's F12 Medium (Ham's F12) containing antibiotics and fungicides. One end of the trachea was ligated with size 000 silk and the other end was connected to sterile plastic tubing. After a 1% pronase solution in Ham's F12 was infused into each trachea, the end of the plastic

tubing was sealed by heated hemostatic forceps. The trachea was incubated at 4°C in Ham's F12 overnight. The epithelial cells were harvested by washing each trachea with 2.5 ml Ham's F12 containing 10% fetal bovine serum (FBS). After centrifugation ($1000 \times g$ for 5 min), the cells were resuspended and filtered through 100 μm nylon mesh to remove large clumps. The cells were plated in a collagen-coated tissue culture dish for the transformation assay. EGVs were counted in 8–10 days and transformation efficiency was calculated in 5 weeks. Cells isolated from EGVs were subcultured for at least 20 passages. The growth or maintenance medium (F12-SM) for these cells consisted of Ham's F12 with the addition of 2% FBS, 15 mM HEPES, 1% L-glutamine, 10 μg/ml insulin, 0.1 μg/ml hydrocortisone, and 0.5% penicillin/streptomycin [(10,000 U)/(10 mg)/ml]. Cells were cultured at 37°C with 5% CO_2 until 50–60% confluent, then isolated following incubation with 1 ml of 0.02% EDTA and 0.25% trypsin in phosphate-buffered saline (PBS). Four milliliters of 10% FBS in Ham's F12 was used to stop the enzymatic activity. After centrifugation ($1000 \times g$ for 5 min), the cells were rinsed in PBS and centrifuged again. Approximately $0.1–1.0 \times 10^6$ cells/ml were resuspended in F12-SM medium and plated in a regular tissue culture dish. More cells were needed in the early passages than that of the later passages for establishment of cell culture.

Normal RTE cells were used as negative controls for anchorage independent-growth and tumorigenicity experiments. At the end of the experiments, the cell lines were tested for *Mycoplasma* contamination using MycoTect Kit (Life Technologies, Grand Island, NY).

2.2. Anchorage-independent growth

Experiments were performed by following Macpherson's method [24] with minor changes. For preparing the 6% agar stock solution, 6 g Agar Nobel was dissolved in 100 ml PBS and autoclaved at 20 psi for 15 min (in liquid cycle), then the bottle was kept in a waterbath at 49°C. Bottom agar was prepared by mixing F12-SM with 6% agar stock at a ratio of 9:1 to make a 0.6% solution of agar. Five milliliters of 0.6% bottom agar was poured into a 60-mm petri dish and incubated at 37°C with 5% CO_2 overnight. Condensate fluid was removed before the top agar was poured. To make top agar, 1 vol. of cell suspension in F12-SM at 37°C was mixed with 9 vols. of 0.33% agar medium (as prepared above) in a waterbath at 47°C. Six milliliters of top agar containing 1.5×10^5 cells was poured onto the top of the bottom agar. Six dishes were used for each cell line. The cultures were kept at 37°C with 5% CO_2 for 4 weeks with no medium change. Colonies containing > 50 cells were counted at the end of each experiment under a phase-contrast microscope. Experiments were performed twice at passages 20 and 30, respectively. CFE was calculated as follows:

$$\text{CFE}\,(‰) = \frac{\text{Number of the colonies}}{\text{Number of the cells plated}} \times 1000.$$

A Poisson test was performed to determine the statistical difference between the results from cell lines induced by DBP and 1-NP and the repeated experiments.

2.3. Tumorigenicity in athymic nude mice

Thirty-eight 3-week-old athymic, nu/nu female mice and four 3-week-old, heterozygote +/nu female mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). The mice were grouped randomly and identified by ear marking. Three nu/nu mice were used for each cell sample, and two were used for medium control. The cells injected into the nude mouse were at the 25th passage. Both sites of the axillary region of the nude mice were injected subcutaneously with 2×10^6 cells in 0.2 ml F12-SM. The residue of cell suspension (0.1 ml) was plated in 25 mm^2 flasks to monitor for contamination and confirm cell survival after introduction into the nude mice. Sterile technique was used for all procedures involving nude mice. They were housed in laminar flow units in a facility fully accredited by the American Association for the Accreditation of Laboratory Animal Care. Cages and sterilized-drinking water were changed twice a week. Heterozygote +/nu mice were used as room and bedding controls for serosurveillance of rodent pathogens (Complete Health Monitoring Profile, Charles River Laboratories, Wilmington, MA).

Mice were monitored daily for general health and to assure that the tumors did not interfere with normal activities. Mice were screened once a week for the appearance and size of tumors. When the size of a tumor reached 10–15 mm in diameter or when 6 months had passed since injection, the mice were sacrificed. Tumors were sliced into three portions: one portion was fixed in 10% neutral buffered formalin (NBF) for histopathologic studies, one was minced and trypsinized for cell cultures to obtain cell lines from the tumors and the third portion was frozen for further studies. If there was no visible mass, the skin around the injection site was still removed and fixed in 10% NBF for histopathological studies. In addition, the lung was inflated with 10% NBF for histopathologic studies.

Tissues in 10% NBF were routinely processed and embedded in paraffin within 24 h of fixation. Sections were stained with hematoxylin and eosin. Skin masses were classified as squamous cell carcinomas (SCC) or squamous cysts (SC). The left lung lobe was examined for histological evidence of metastases. All histological assessments were conducted by a veterinary pathologist blinded to the inoculum used.

The results of tumorigenicity were calculated as follows:

$$\text{Percentage of SCC} = \frac{\text{Number of the sites displaying SCC}}{\text{Number of the sites inoculated}} \times 100$$

$$\text{Percentage of SC} = \frac{\text{Number of the sites displaying SC}}{\text{Number of the sites inoculated}} \times 100.$$

A χ^2 analysis was used to compare the results of the cell lines induced by 1-NP and DBP.

3. Results

3.1. Anchorage-independent growth

As Table 2 shows, normal RTE cells and the two cell lines from spontaneous foci did not grow in soft agar. All five of the cell lines from foci induced by 1-NP and three of the five cell lines from foci induced by DBP possessed the ability to grow in soft agar. There is no significant difference between results of the 20th and the 30th passage ($P > 0.05$). In the experiment for the 20th passage, there were 300 colonies in total from 4.5×10^6 cells treated by DBP and the CFE was 0.067‰. There were 678 colonies in total from 4.5×10^6 cells treated by 1-NP and the CFE was 0.151‰. The difference between the CFE of cells induced by DBP and 1-NP is statistically significant ($\mu = 12.08$, $P < 0.01$).

Table 2
Colony forming efficiency (CFE) of RTE cell lines in anchorage-independent growth experiments

Samples	The 20th passage (‰)	The 30th passage (‰)
DB1[a]	0.10	0.07
DB3	0	0
DB4	0	0
DB18	0.16	0.15
DB19	0.11	0.094
NP1[b]	0.25	0.25
NP2	0.16	0.16
NP3	0.04	0.03
NP4	0.26	0.21
NP14	0.04	0.03
Spon.1[c]	0	0
Spon.2	0	0
Normal RTE[d]	0	0

[a] DB1–DB19, cell lines from foci induced by DBP.
[b] NP1–NP14, cell lines from foci induced by 1-NP.
[c] Spon.1 and spon.2, cell lines from foci spontaneously occurred.
[d] Normal rat tracheal epithelial cells.

3.2. Tumorigenicity in athymic nude mice

Each mouse was injected at two sites. The earliest mass in nude mice was observed 10 days after injection of transformed RTE cells. Mice were sacrificed when the tumor size reached about 15 mm. This occurred at 1.8 months (56 days) for the first mouse injected with 1-NP-induced-transformed cells and at 2.7 months (83 days) for the first mouse injected with DBP-induced-transformed cells. In 15 mice inoculated with DBP transformed cell lines, five mice exhibited SCCs at six sites, yielding a frequency of SCC of 20% (6/30). Three mice had SCs at three sites yielding a frequency of SC of 10% (3/30). Two of five DBP transformed cell lines produced SCC and two other DBP transformed cell lines produced SC. In 15 mice inoculated with 1-

Table 3
Summary results of tumorigenic potential of DBP-and 1-NP-induced RTE cell lines in nude mice

Cell lines	No. of mice injected	No. of mice exhibiting SCC[a]	Sites of SCC inoculations (%)	No. of mice exhibiting SC[b]	Sites of SC inoculations (%)
DB1	3	2	2/6 = 33	0	0/6 = 0
DB3	3	0	0/6 = 0	0	0/6 = 0
DB4	3	0	0/6 = 0	1	1/6 = 17
DB18	3	3	4/6 = 67	0	0/6 = 0
DB19	3	0	0/6 = 0	2	2/6 = 33
Subtotal	15	5	6/30 = 20	3	3/30 = 10
NP1	3	3	6/6 = 100	0	0/6 = 0
NP2	3	2	4/6 = 67	1	1/6 = 17
NP3	3	1	1/6 = 17	1	1/6 = 17
NP4	3	3	4/6 = 67	1	1/6 = 17
NP14	3	2	2/6 = 33	0	0/6 = 0
Subtotal	15	11	17/30 = 57**	3	3/30 = 10
Spon.1	3	0	0/6 = 0	1	1/6 = 17
Spon.2	3	0	0/6 = 0	2	4/6 = 67
Subtotal	6	0	0/12 = 0	3	5/12 = 42
Normal RTE	3	0	0/6 = 0	0	0/6 = 0
Medium	2	0	0/4 = 0	0	0/4 = 0
Subtotal	5	0	0/10 = 0	0	0/10 = 0

[a] Squamous cell carcinoma.
[b] Squamous cyst.
** $P < 0.01$.

NP-transformed cell lines, 11 mice exhibited SCC at 17/30 (57%) injection sites. Three mice exhibited SCs at 3/30 (10%) injection sites. All five 1-NP-transformed cell lines produced SCC and three of them produced SC. None of the six mice inoculated with cells from spontaneously occurring foci exhibited SCC, but three of them developed SC at 5/12 (42%) injection sites. There is a significant difference between the frequencies of SCC formed by cell lines induced by the different compounds ($P < 0.01$), but there is no significant difference in the frequencies of SC formed by the cell lines. None of the mice inoculated with normal RTE cells or F12-SM control medium exhibited SCC or cysts (see Table 3), whereas, one of the four sites injected with medium control exhibited a small mast cell tumor (a spontaneous tumor of mice). The tumor was examined histopathologically and was morphologically consistent with a round cell tumor. Histochemical staining with toluidine blue revealed numerous cytoplasmic metachromatic granules confirming the diagnosis of a mast cell tumor. No metastatic foci were observed in the lungs of mice inoculated with RTE cells. The inoculated RTE cells showed no evidence of *Mycoplasma*. Immunocompetent room and bedding surveillance mice were seronegative for rodent pathogens, indicating that the experiments were not compromised by any pathogens.

4. Discussion

Thomassen et al. [25] stated that (1) EGV colonies contained heterogeneous cell populations of dividing (stem) and non-dividing (non-stem) cells; (2) the percentage of the stem cells in one focus varied from 5–50%; (3) in one EGV focus not all the stem cells were transformed, only the stem cells actually transformed could grow on plastic surface, and only these could be subcultured to become immortal cell lines. In our studies, only plastic dishes were used for cell line establishment, therefore, only the cell populations which were transformed stem cells were selected.

Our previous studies showed that EGVs were induced in the primary cultures of RTE cells exposed in vivo by either DBP or 1-NP (see Table 1). However, 1-NP-induced EGVs possessed a higher capacity of becoming cell lines (25/48) than those induced by DBP (8/28)($\chi^2 = 4.0$, $P < 0.05$). This may indicate that DBP-induced EGVs contain less

transformed stem cells and the stem cells are less transformed than those induced by 1-NP. Under a phase-contrast microscope, the transformed cells induced by DBP were morphologically different than those induced by 1-NP. The transformed cells induced by DBP exhibited relatively larger size and lower ratio of nucleus: cytoplasm than those induced by 1-NP. Also, the cells from EGVs induced by DBP grew slower than those from EGVs induced by 1-NP.

Results of the anchorage-independent growth assay showed that two of five from DBP- and five of five from 1-NP-induced cell lines displayed anchorage-independent growth and the cells from DBP-induced RTE cell lines had lower CFE (0.067‰) than those induced by 1-NP (0.151‰) ($\mu = 12.08$, $P < 0.01$). Normal RTE cells and cells from either of the two spontaneously transformed cell lines did not grow in soft agar. It has been stated that once RTE cell lines show the characteristics of anchorage-independent growth, the CFE in soft agar tends to increase with increasing passage number [26]. In this study, however, cells of the 20th and 30th passages did not show any significant difference in the frequency of anchorage-independent growth for RTE cell lines.

In the tumorigenicity experiments, the tumors found in these studies are classified as malignant (SCC) or benign (SC). Two of five DBP- and five of five 1-NP-induced cell lines exhibited SCC in nude mice. The difference between the frequencies of SCC from the DBP- ($6/30 = 20\%$) and 1-NP-induced ($17/30 = 57\%$) transformed RTE cell lines is statistically significant ($\chi^2 = 8.53$, $P < 0.01$). Neither of the normal RTE cells nor the ones from spontaneously transformed cells induced SCC. Cells from spontaneously occurring RTE cell lines produced only cysts ($5/12 = 41\%$). Some of the RTE cells from DBP- ($3/30 = 10\%$) or 1-NP-induced cell lines ($3/30 = 10\%$) also exhibited SC which were whitish, remained small and were filled with keratin and cell debris. There is no significant difference among the incidences of SC from different cell lines ($\chi^2 = 0.08$, $P > 0.05$).

The tumorigenicity of the RTE cell lines in nude mice correlated well with the results from the anchorage-independent growth assay. Fourteen of the 26 mice that exhibited tumors had tumors on only one site and the size of tumors in one individual at different sites were sometimes different. This phenomenon is consistent with the fact that the immunoactivity of nude mice increases as the nude mice mature [27]. In this study, it is possible that a small proportion of transformed cells possessing neoplastic properties grew relatively slowly and the immunoactivity of the nude mice, e.g., non-T-cell-dependent NK cells, increased as they grew. The increased immune activity suppressed the growth of transformed cells on the other side injected with the cells. Another finding in these studies is that one of the four sites injected with culture medium as control exhibited a mast cell tumor. The mass was so small that it could not be seen with the naked eye and the mass was not of tracheal epithelial origin. This phenomenon indicates that it is possible that nude mice could generate spontaneous tumors at injured sites, e.g., by needle injection or that this was a chance event at the inoculation site.

Based on the limited number of RTE cell lines studied, it seems that cell lines induced by 1-NP possess stronger neoplastic potential than those induced by DBP because the time for reaching a specific size of tumors is shorter and the number of SCC, as well as the CFE in anchorage-independent growth experiments, are statistically higher for the transformed cells induced by 1-NP than those induced by DBP. The reason for the different neoplastic potentials of the cell lines induced by those two compounds is not known. Further studies are needed to determine whether these chemicals cause different types of genetic damage in RTE cells.

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