Differential Induction of Squamous Cell Carcinomas and Adenocarcinomas in Mouse Lung by Intratracheal Instillation of Benzo(a)pyrene and Charcoal Powder

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ABSTRACT

Differential induction of squamous cell carcinomas, adenomas, and adenocarcinomas was observed in the lungs of male C57BL/6 and C3H/He mice after repeated intratracheal instillation of benzo(a)pyrene (BP) and charcoal powder suspended in 0.9% NaCl solution. When a high dose of BP (1.0 mg BP and 0.5 mg charcoal powder) was instilled intratracheally once a week for 8 weeks or when a low dose of BP (0.5 mg BP and 0.5 mg charcoal powder) was instilled once a week for 16 weeks, squamous cell carcinomas were induced in high incidence (77 to 87%) in the early period of observation, whereas pulmonary adenomas and adenocarcinomas were induced in low incidence (0 to 48%) in the late period of observation in both strains of mice. On the other hand, when a low dose of BP was instilled intratracheally once a week for 8 weeks, pulmonary adenomas and adenocarcinomas were induced in high incidence (76 to 91%), but squamous cell carcinomas were induced in low incidence (9 to 26%). These results show that a larger quantity of BP instilled intratracheally was needed for induction of squamous cell carcinomas than for induction of adenomas and adenocarcinomas in the lung of mice. Thus, when the carcinogen is administered to a single organ of a single mouse strain by the same route, different amounts of carcinogen have different effects on the incidences of various histological types of tumors.

INTRODUCTION

There have been many studies over many years on induction of experimental lung tumors in mice by chemical carcinogens, and results have been reviewed (21, 23). Most of the pulmonary tumors induced by chemical carcinogens in mice have been pulmonary adenomas. These pulmonary adenomas develop spontaneously in aged mice of various strains, and they can also be induced by i.v., i.p., or s.c. injections of polycyclic hydrocarbons (1, 3), urethane (16, 22), 4-nitroquinoline 1-oxide (13, 14), N-nitroso compounds (12, 25), and other chemicals (21, 23).

There have, however, been few reports about the induction of squamous cell carcinomas in lungs of mice by chemical carcinogens. In 1937, Andervont (1) reported that squamous cell carcinomas are inducible by insertion of 1,2,5,6-dibenzanthracene-impregnated strings into the lungs of mice, but the incidence of these tumors was low. Kotin and Wiseley (11) reported a method for the induction of squamous cell carcinomas in mouse lungs by inhalation of influenza virus and aerosols of hydrocarbons, but the incidence of these tumors was also rather low. Successful induction of squamous cell carcinomas by repeated i.t. administration of a carcinogen to mice, as well as to hamsters (2, 4, 18), rats (17, 19, 20), and rabbits (6, 7), was reported by Nettesheim and Hammons (15) and by Ho et al. (8, 9).

In a previous report (27), we showed that squamous cell carcinomas could be induced rapidly and in high incidence by repeated i.t. instillation of BP and charcoal powder into the lungs of C57BL/6 mice. C57BL/6 mice were used in these studies because they are considered to show the lowest incidence of spontaneous pulmonary adenomas (23), and BP and charcoal powder were selected because they are naturally present in our environment. This report describes studies on the effect of the dose and time of BP instillation on the inductions of squamous cell carcinomas, adenomas, and adenocarcinomas in the lungs of 2 mouse strains, C57BL/6 and C3H/He.

MATERIALS AND METHODS

Animals. Ten-week-old male C57BL/6 and C3H/He mice were supplied from the Funabashi Animal Farm (Kyoto, Japan). They were given free access to standard animal diet (NMF Rat and Mouse Diet; Oriental Yeast Co. Ltd., Chiba, Japan) and water.

Preparation of Carcinogens. A mixture of BP (3,4-benzo-pyrene; Sigma Chemical Co., St. Louis, Mo.) and charcoal powder (Wako Pure Chemical Ind., Ltd., Osaka, Japan) in a ratio of 2:1 or 1:1 was ground thoroughly in an agate mortar for about 1 hr. The mixture was stored in a glass bottle in the dark at room temperature until use. Sterile 0.9% NaCl solution was added to the mixture to give a final concentration of 40 mg BP and 20 mg charcoal powder or 20 mg BP and 20 mg charcoal powder per ml 0.9% NaCl solution. This suspension was further homogenized in a Teflon homogenizer with a loosely fitting pestle, transferred to a sterile glass bottle, and kept homogeneous by continuous stirring during the procedure of i.t. instillation.

Instillation i.t. The suspension was instilled i.t. as described previously (27). Briefly, each mouse was anesthetized with ether and fixed with rubber bands in a supine position on a board. The tongue was pulled outward and displaced laterally.

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2 To whom all requests for reprints should be addressed.
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The abbreviations used are: i.t., intratracheal; BP, benzo(a)pyrene.
with small forceps, and saliva was wiped away with a sterile paper string. The vocal cords were located with a frontal mirror, then the 22-gauge blunt needle of a tuberculin syringe was inserted slowly into the trachea, and the suspension was injected slowly from about the position of bifurcation of the trachea. Mice were given a high dose of BP (1.0 mg of BP and 0.5 mg of charcoal powder) or a low dose of BP (0.5 mg of BP and 0.5 mg of charcoal powder) in 0.025 ml of 0.9% NaCl solution i.t. once a week for 4, 8, or 16 weeks. Control mice were given 0.5 mg of charcoal powder in 0.025 ml of 0.9% NaCl solution once a week for 8 weeks.

Histological Examinations. The mice were examined daily and weighed weekly throughout the observation period of 120 weeks. Animals that died naturally or were killed when they became moribund were autopsied. The lungs were excised en bloc with the trachea and mediastinal organs, liver, spleen, kidneys with adrenals, and stomach. Tissues were fixed in 10% Formalin solution, sectioned at 5 μm thickness, and stained with hematoxylin and eosin.

For examination of the distribution of BP in the lung, 5 mice were killed 1 hr after i.t. instillation of BP, and cryostat sections of the lung were examined in a fluorescence microscope. Some specimens were also stained with hematoxylin and eosin and observed microscopically. For histological examination of early changes of the epithelium of the respiratory tract, 5 mice were killed serially 1, 3, 5, 7, and 10 weeks after receiving 8 high doses of BP.

RESULTS

Changes in Mean Body Weight and Survival Rate. Changes in mean body weight and the cumulative survival rates of experimental and control animals are shown in Table 1. The mean body weights of the mice given a high dose of BP i.t. once a week for 8 weeks decreased greatly during treatment but increased gradually after treatment. On the other hand, the mean body weight of mice treated with 8 to 16 low doses of BP increased steadily during treatment. These results show that instillation of a high dose of BP i.t. had a toxic effect but that instillation of a low dose did not. The life span of mice treated with a high dose of BP was considerably less than that of mice treated with a low dose of BP. Mice treated 16 times with a low dose of BP had an intermediate life span. Some of the control mice died of pneumonia without tumors during the observation period.

Macrosopic Observations. Charcoal powder was distributed almost equally in each lobe of the lung after a single i.t. instillation of BP and charcoal powder. In both C57BL/6 and C3H/He mice treated 8 times with a high dose of BP, many tiny nodular lesions with diameters of about 0.5 to 2.0 mm were observed on the surface of the lung and on the cut surface of the lung from as early as 2 weeks after the last instillation. These nodular lesions were always surrounded by charcoal deposits.

In mice treated 8 times with a high dose of BP, tumors of various sizes were observed after Week 10 of the observation period. Usually, these tumors were solitary, but sometimes 2 or more tumors were found. The tumors found in the early period of observation were all squamous cell carcinomas. Most of the tumor nodules were seen as round, hard, white swellings on the surface of the lung (Fig. 1A). The tumors had an indistinct, irregular outline. Sometimes, tumors were found at the main bronchus, and the cut surface of the tumor showed the stenotic bronchus surrounded by a tumor mass. Occasionally, squamous cell carcinomas were disseminated in the pleural cavity (Fig. 1B) or had metastasized to the kidneys. Usually, pulmonary adenomas of the lung were multiple. These tumors were seen on the surface of the lung as round or regular opaque swellings, and they were softer than squamous cell carcinomas. Small tumors were 1 to 2 mm in diameter, while general, squamous cell carcinomas were disseminated in the pleural cavity or had metastasized to the kidneys. Occasionally, squamous cell carcinomas were disseminated in the pleural cavity (Fig. 1B) or had metastasized to the kidneys. Usually, pulmonary adenomas of the lung were multiple. These tumors were seen on the surface of the lung as round or regular opaque swellings, and they were softer than squamous cell carcinomas. Small tumors were 1 to 2 mm in diameter, while many tiny nodular lesions with diameters of about 0.5 to 2.0 mm were observed on the surface of the lung and on the cut surface of the lung from as early as 2 weeks after the last instillation. These nodular lesions were always surrounded by charcoal deposits.

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<td>BP (1.0 mg), charcoal powder (0.5 mg), 8 instillations</td>
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* Numbers in parentheses, number of survivors.
large tumors were about 10 mm in diameter. Occasionally, adenocarcinomas were disseminated in the pleural cavity with massive pleural effusion, or they had metastasized to the kidneys.

**Microscopic Findings.** Fluorescent microscopy showed crystals of BP almost evenly distributed in the periphery of the lung, and especially in the terminal bronchioles and alveoli. Sections of lung stained with hematoxylin-eosin showed charcoal powder distributed in the same way as was BP and occasionally phagocyted by alveolar macrophages, which formed giant cells of various sizes.

One week after instillation of 1.0 mg BP and 0.5 mg charcoal powder, microgranulomas composed of histiocytes and epithelioid cells were found scattered in the lung. At Week 3, hyperplastic changes and squamous metaplasia of the epithelium of terminal bronchioles was usually observed. At Week 5, proliferative changes and squamous metaplasias in terminal bronchioles were seen. At the same time, squamous metaplasias were also observed in the epithelium of the central bronchi and trachea. In Week 7, moderately to highly keratinized microtumors were observed in the lung. Some of them were about 2 mm in diameter and were composed of squamous tumor cells with prominent nucleoli.

After Week 10, tumors of various sizes were observed. Some of them were highly keratinized squamous cell carcinomas. Islands of tumor cells were seen scattered in the massive layers of keratinization. Pearl formation was usually seen. Moderately keratinized squamous cell carcinomas were also observed. Tumor cells had intercellular bridges and oval nuclei with frequent mitoses (Fig. 2A). Poorly differentiated squamous cell carcinomas were rarely seen. These tumors often contained central foci of necrosis (Fig. 2B) and were composed of anaplastic cells with little keratinization. Invasion of squamous cell carcinomas into blood vessels was occasionally seen, but metastases to regional lymph nodes and distant organs were rare. Metastatic tumors in the kidneys were moderately keratinized or poorly differentiated squamous cell carcinomas (Fig. 2C).

Pulmonary adenomas and adenocarcinomas were induced later during the observation period than were squamous cell carcinomas. Pulmonary adenomas were tentatively classified into alveolar-type adenomas and papillary-type adenomas. The alveolar-type adenomas were 100 μm to 2 mm in diameter. These tumors grew slowly and did not compress adjacent lung tissue appreciably (Fig. 3A). These tumors consisted of uniformly cuboid cells containing uniform nuclei, and they tended to grow along the alveolar septa. Development of the stroma was scanty. Growth of papillary-type adenomas was moderate. Most of these tumors showed papillary rather than cystic growth with little stroma (Fig. 3B). The tumors were composed of rather uniformly cuboid or cylindrical cells, but their nuclei were not always uniform. Some mitoses were seen.

Well-differentiated adenocarcinomas grew rapidly, forming irregular glandular structures. These tumors were composed of irregular cells with chromatin-rich nuclei, frequent mitoses, and well-developed stroma. Most of the primary adenocarcinomas were found in the peripheral or central part of papillary-type adenomas (Fig. 3C). Some poorly differentiated adenocarcinomas were also seen (Fig. 3D). These tumors had poorly organized glandular structures and had a sarcoma-like appearance. They were often composed of spindle-shaped, polygonal cells. The adenocarcinomas frequently showed invasion of adjacent organs and metastasis to the kidneys. Metastatic adenocarcinomas often had a sarcoma-like appearance (Fig. 3E).

**Incidence of Pulmonary Tumors.** The incidences of tumors and other findings in the lung of C57BL/6 and C3H/He mice are shown in Charts 1 and 2. Mice that died during ether anesthesia, from pneumonia during treatment, or from cannibalism are not included. In the group of C57BL/6 mice treated with a high dose of BP once a week for 8 weeks, squamous tumors were not always uniform. Some mitoses were seen.

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cell carcinomas were induced in the early period of observation, and their incidence was 34 of 44 (77%). Pulmonary adenomas were observed in the late period of observation, and their incidence was 2 of 44 (5%). One squamous cell carcinoma was observed in the trachea. In the group of C3H/He mice treated 8 times with a high dose of BP, the incidence of squamous cell carcinomas was high [16 of 20 (80%)]. Two squamous cell carcinomas were found in the trachea.

In the group of C57BL/6 mice treated 8 times with a low dose of BP, squamous cell carcinomas were induced in only 10 of 38 animals (26%), but pulmonary adenomas were induced in high incidence. The incidence of alveolar-type adenomas was 8 of 38 (21%), that of papillary-type adenomas was 18 of 38 (47%), and that of adenocarcinomas was 3 of 38 (8%). In the group of C3H/He mice treated 8 times with a low dose of BP, squamous cell carcinomas were induced in 3 of 35 animals (9%). In this group, the incidences of pulmonary adenomas were high in the late period of observation. The incidence of alveolar-type adenomas was 6 of 35 (17%), that of papillary-type adenomas was 20 of 35 (57%), and that of adenocarcinomas was 6 of 35 (17%). Three of 6 adenocarcinomas were accompanied with bloody pleural effusion and dissemination of tumor nodules to the chest wall or kidney metastasis.

In the group of C57BL/6 mice treated 16 times with a low dose of BP, squamous cell carcinomas were induced in high incidence [22 of 27 (81%)]. Pulmonary adenomas and adenocarcinomas were rare and were observed only late in the observation period. In the group of C3H/He mice treated 16 times with a low dose of BP, squamous cell carcinomas were induced in high incidence [27 of 31 (87%)], but the incidence of adenomas was low [4 of 31 (13%)]. In the group of C3H/He mice treated 4 times with a low dose of BP (not shown in Chart 2), pulmonary adenomas were induced in 7 of 9 animals and one adenocarcinoma was induced after between 61 and 81 weeks, but there were no squamous cell carcinomas.

In the 34 control C57BL/6 mice treated 8 times with 0.5 mg of charcoal powder, no tumor was found within 110 weeks. In the 33 control C3H/He mice, only one alveolar-type adenoma was observed within 100 weeks.

**DISCUSSION**

Differential induction of squamous cell carcinomas, adenomas, and adenocarcinomas was observed in the lungs of 2 mouse strains, C57BL/6 and C3H/He, by repeated i.t. instillation of BP and charcoal powder. Pulmonary adenomas developed spontaneously in various mouse strains, but strain C3H/He is relatively resistant and strain C57BL/6 is very resistant to development of spontaneous pulmonary adenomas (23). We found that the incidence of pulmonary adenomas and adenocarcinomas was about 75 to 90% in both strains after i.t. instillation of a low dose of BP for 8 weeks. We also found that squamous cell carcinomas were induced in high incidence by i.t. instillation of a high dose of BP weekly for 8 weeks or a low dose of BP weekly for 16 weeks in both mouse strains. Therefore, this model system should be useful for studies not only on the development of pulmonary adenomas and adenocarcinomas but also on the development of squamous cell carcinomas in the lung.

Our results showed a relationship between the dose of BP and the induction of pulmonary tumors in mice and also differences in induction periods of squamous cell carcinomas, adenomas, and adenocarcinomas. A larger dose of BP was necessary for induction of squamous cell carcinomas than for induction of adenomas and adenocarcinomas. Thus, it seems that on administration of a carcinogen to a single organ of a single mouse strain by the same route, the amount of the carcinogen affects the incidences of different histological types of tumors. Consistent with our findings, Nettesheim and Hammons (15) reported that squamous cell carcinomas were induced rapidly in high incidence in the lungs of C57BL/6 x C3H F1 mice by repeated i.t. instillation of 3-methylcholanthrene in gelatin solution but that no pulmonary adenomas and adenocarcinomas were induced. In contrast, Ho et al. (8, 9) reported that squamous cell carcinomas and adenomas or adenocarcinomas were induced in the lung of 3 mouse strains, C57BL/6, DBA/2J, and NIH-Swiss/Mai, by repeated i.t. instillation of different amounts of BP or 3-methylcholanthrene suspended in gelatin solution but that no squamous cell carcinomas was induced within 7 months in the lung of C57BL/6J mice by i.t. instillations of 0.5 mg BP. The difference between these results and ours is possibly due to a different observation period or a different clearance rate of BP. We did not examine the clearance rate of BP, but the clearance of BP administered with charcoal powder in 0.9% NaCl solution is probably less than that of BP suspended in gelatin solution.

In the experiment on serial killing of C57BL/6 and C3H/He mice treated with a high dose of BP, we consistently observed squamous metaplasias in the epithelium of the trachea, bronchi, and bronchioles from as early as 3 weeks after the first instillation of BP. The incidence of squamous metaplasias of the epithelium of terminal bronchioles in particular was very high. Probably most of the squamous cell carcinomas of the lung of mice originate in the epithelium of the bronchioles. However, some squamous cell carcinomas were clearly related to large bronchi, and a few squamous cell carcinomas were observed in the trachea. These observations were rather different from those on hamsters (18), in which the incidence of tracheal tumors was high. Our results indicate that most squamous cell carcinomas of mouse lungs originate in the bronchiole, as in rats (19), but that a few originate in the bronchi and in the trachea. The incidences of tumors at different sites may be related to the properties of the BP suspension instilled i.t. Judging from the distributions of BP and charcoal powder in the lung, the terminal bronchioles seem to be the main site of deposition of BP and charcoal powder, and squamous cell carcinomas originated in this region.

As shown in Charts 1 and 2, the incidence of squamous cell carcinomas in the trachea was low. Squamous metaplasias were regularly induced in the epithelium of the trachea after i.t. instillations of 1.0 mg BP and 0.5 mg charcoal powder for 3 or 5 weeks, but the incidence of squamous metaplasias of the epithelium of the trachea was low. Similarly, multiple squamous metaplasias were consistently observed in the terminal bronchioles of the lung, but the number of squamous cell carcinomas in the lung was usually only one, or occasionally 2 or 3. These results suggest that most of the squamous metaplasias were toxic reversible changes induced by BP and that only some of them were irreversible, developing into squamous cell.
carcinomas. It is difficult to predict from these studies what kind of squamous metaplasia will develop into a carcinoma. The early mortality of mice treated with a high dose of BP (Table 1) may be related to generalized squamous metaplasia of the respiratory tract.

There are several reports (3, 13) on the histogenesis of pulmonary adenomas in mice induced by chemical carcinogens. It is generally thought that pulmonary adenomas in mice progress into adenocarcinomas. We tentatively classified these tumors into alveolar-type adenomas, papillary-type adenomas, and adenocarcinomas according to their growth pattern, development of stroma, and cellular irregularity. We observed that most adenocarcinomas were present in the peripheral or central part of papillary-type adenomas and that many adenocarcinomas in invasive or metastatic sites were composed of spindle cells. Similar histological findings have been reported for metastatic adenocarcinomas originating spontaneously in mice (26), for adenocarcinomas induced by 4-nitroquinoline 1-oxide (13), and for tumors developing from s.c.-transplanted pulmonary adenomas (10, 24). Probably, most of the adenocarcinomas of the lung of mice are derived from papillary-type adenomas and progress into spindle cell carcinomas.

In this work, we found that the i.t. instillation method is useful even in mice. Mice are the most commonly used experimental animals in studies on immunogenetics. Pulmonary adenomas in mice have been analyzed genetically by Heston (5), but metastases developing from s.c.-transplanted pulmonary adenomas (10, 24). Probably, most of the adenocarcinomas of the lung of mice are derived from papillary-type adenomas and progress into spindle cell carcinomas.

ACKNOWLEDGMENTS

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REFERENCES


Experimental Lung Cancer in Mice
Fig. 1. A, gross appearance of a large squamous cell tumor in the lower lobe of the left lung. Note the irregular outline of the tumor. C3H/He, low dose of BP, 16 times; experimental period, 299 days. B, gross appearance of multiple metastatic tumor nodules on the chest wall. C3H/He, low dose of BP, 16 times; experimental period, 300 days.

Fig. 2. A, moderately keratinized squamous cell carcinoma. Tumor cells had intercellular bridges and oval nuclei with frequent mitoses. C3H/He, low dose of BP, 8 times; experimental period, 200 days. H & E, x 200. B, poorly differentiated squamous cell carcinoma. This tumor contained central foci of necrosis. Note the irregular growth with little keratinization. C3H/He, low dose of BP, 16 times; experimental period, 229 days. H & E, x 200. C, kidney metastasis of a squamous cell carcinoma. Upper area, metastatic squamous cell carcinoma with necrosis; lower area, proximal convoluted tubules and glomeruli of the kidney. C57BL/6, low dose of BP, 16 times; experimental period, 325 days. H & E, x 100.
Fig. 3. A, alveolar-type adenoma. Tumor cells are uniformly cuboidal with uniform nuclei. C3H/He, low dose of BP, 8 times; experimental period, 344 days. H & E, x 200. B, papillary-type adenoma. Tumor cells are uniformly cuboidal or cylindrical, but their nuclei are not uniform. C57BL/6, low dose of BP, 8 times; experimental period, 711 days. H & E, x 200. C, adenocarcinoma arising in a papillary-type adenoma. An island of pleomorphic tumor cells is seen in a papillary-type adenoma. C57BL/6, low dose of BP, 8 times; experimental period, 737 days. H & E, x 100. D, poorly differentiated adenocarcinoma. Note the glandular structure and polymorphic tumor cells. C3H/He, low dose of BP, 8 times; experimental period, 445 days. H & E, x 200. E, regional lymph node metastasis of moderately differentiated adenocarcinoma. Charcoal deposits can be seen in the lymph node. C57BL/6, low dose of BP, 8 times; experimental period, 670 days. H & E, x 100.
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