Review

Detection of Carcinogenic and Modifying Potentials by Test Compounds Using a Mouse Lung Carcinogenesis Bioassay

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Abstract: Lung cancer is one of the most common cancers in the world, and the incidence of lung cancer is increasing. Therefore, it is particularly important to detect carcinogenic or tumor promoting substances of lung carcinogenesis in our environment, so that such harmful chemicals can be eliminated from our environment. Furthermore, detection of chemopreventive agents of lung carcinogenesis is also important to reduce our risk of lung cancer. It is necessary to establish reliable in vivo animal models of lung carcinogenesis for that purpose. The A/J mouse is a mouse strain sensitive to lung carcinogens, and also develops spontaneous lung tumors without any chemical treatment. In our department, we have demonstrated that a treatment of 4-(methylnitrosamino)-1-(3-pyridyle)-1-butanone (NNK), a tobacco specific nitrosamine, or 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (MeIQx), a heterocyclic amine, induced lung tumors in the female A/J mouse in 16 and 32 weeks. The lung tumors developed in the A/J mouse are histopathologically classified as adenocarcinomas, adenomas, and alveolar cell hyperplasias. Some of these types of lung cancer are similar to those of human lung cancer. We also investigated the chemopreventive effects of bovine LF (bLF) on different phases of NNK-induced lung tumorigenesis in A/J mice. The A/J mouse is very useful mouse strain as a reliable in vivo model, which can be used for detecting chemopreventive agents of lung carcinogenesis.

Key words: lung carcinogenesis, carcinogen, promoter, chemoprevention, mouse, bioassay

Introduction

Lung cancer is one of the main causes of death due to cancer in the United States, Japan and most of other countries. There are several morphologically different types of lung cancer, and non-small-cell lung carcinoma represents about 80% of overall lung cancer cases worldwide¹. In order to identify hazardous compounds or risk factors in our environment, it is very important to use suitable animal experimental bioassay models. In fact, there are many good animal models for some organ carcinogenesis bioassays, such as the liver²,⁴, stomach⁴, and urinary bladder⁵,⁶. However, in terms of lung carcinogenesis bioassay models, there are no good models for predicting lung carcinogens, and promoting or chemopreventive compounds. Therefore, it is particularly important to detect carcinogenic or tumor promoting substances of lung carcinogenesis in our environment, so that such harmful chemicals can be removed from our environment. Furthermore, the detection of chemopreventive agents of lung carcinogenesis is also important to reduce our risk of lung cancer. It is necessary to establish reliable in vivo animal models of lung carcinogenesis for that purpose.

In this review, we summarize some experiments using lung carcinogenesis models, mainly mouse lung carcinogenesis models which have mostly performed in our department.

Effects of Dietary Factors on Lung Carcinogenesis

Many dietary factors have been reported to modify carcinogenesis in different organs of man and experimental animals⁷–¹¹. In animal models, high fat diets have been demonstrated to promote mammary¹²–¹⁴, colon¹⁵, prostate¹⁶,¹⁷ and pancreas carcinogenesis¹⁸, and in man, high fat diet food intake has also been associated with elevated relative risk of colon cancer development¹⁹. With regard to lung carcinogenesis, only a few promoters have been identified, such as bleomycin¹⁹, butylated hydroxytoluene²⁰–²², glycerol²³,²⁴ or iron administration²⁵. We investigated the influence of dietary high fat diet on 4-nitroquinoline 1-oxide (4NQO)-induced
lung tumorigenesis in the ICR mouse26.

A total of 160, 6-week-old male ICR mice (Charles River Japan Inc., Atsugi) were divided into 4 equal groups: Groups 1 and 2 were given a single injection of 4NQO subcutaneously at a dose of 15 mg/kg body weight, and Groups 3 and 4 received a single injection of 10 ml/kg body weight of the oil mixture without 4NQO. One week later, Groups 1 and 3 were placed on a diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo: 3.5 kcal/g) containing 20% corn oil (high fat diet; 4.7 kcal/g), the main components of the CRF-1 diet and the corn oil being as follows: oleic acid (22.4: 32.8%), linoleic acid (50.2: 51.9%), linoleic acid (4.6: 1.8%). Groups 2 and 4 were maintained on basal diet without supplement.

Ten mice from each group were sacrificed at weeks 15 and 18, and all surviving animals (19 to 20 mice per group) were sacrificed at week 25. At each time point, all lobes of each lung were examined using a stereomicroscope, and lesions found were examined histopathologically.

The incidence of lung lesions in group 1 (4NQO-Fat) were always higher than in group 2 (4NQO), and although the differences were not significant at each time point, Petos’s trend test revealed a significant difference between the two groups overall. Furthermore, the mean numbers of lung tumors/animal were significantly different between groups 1 and 2 sacrificed at weeks 15 and 25. This study demonstrated that dietary high fat clearly enhances the development of 4NQO-initiated mouse lung tumors26. Similar results were also published for benzo[a]pyrene-induced hamster respiratory tumor development37 and N-nitrososobis(2-oxopropyl)amine-induced hamster lung tumorigenesis38.

Other dietary factors of lung carcinogenesis were reported for heterocyclic amines, mutagenic compounds found in cooked foods, especially 2-amino-3,8-dimethylimidazolo[4,5-f]quinoline (MeIQx)29,30.

Tobacco-Smoking and Animal Lung Carcinogenesis

Tobacco-smoking has been epidemiologically associated with lung cancer in humans. Experimentally, however, animal models of tobacco-smoking induced lung cancer have not been successful. In a hamster model, larynx tumors including carcinomas were induced by exposure to cigarette smoke. Dontenwill et al. reported that in hamsters exposed nose only to the smoke of various cigarettes, the lesions in the larynx depended on the smoke dose and duration of treatment31. We also reported that mainstream tobacco smoke promoted tracheal tumorigenesis initiated by diethylnitrosamine in Syrian golden hamsters32. However, most hamster models of tobacco-smoking were of larynx or tracheal tumorigenesis, but not of lung carcinogenesis itself. In a rat model, Mauderly et al. recently demonstrated convincing, although moderate, increases in tumors of the lung and nasal mucosa in rats exposed to cigarette smoke33. For dogs and non-human primates as well as hamsters, studies of chronic inhalation studies with mainstream cigarette smoke were reviewed by Coggins34.

Regarding mouse studies of tobacco-smoking, Hutt et al. recently reported that life-span inhalation exposure to mainstream cigarette smoke successfully induces lung cancer in B6C3F1 mice35. After their publication, Hecht reported his commentary and reviewed tobacco-smoking and carcinogenesis, especially regarding animal models for identifying risk factors in mainstream tobacco smoke36. Lung cancer in humans is mainly classified into small cell carcinoma or non-small cell carcinoma, with the non-small cell carcinoma including squamous cell carcinoma, adenocarcinoma and large cell carcinoma. The incidence of lung cancer has been increasing in most countries, especially the incidence of lung adenocarcinomas has increased, particularly among women, and adenocarcinoma has become the most common histological type of lung cancer37. An A/J mouse model that is responsive to cigarette smoke has been described by Witschi et al.38. Lung adenocarcinomas in mouse, including the A/J mouse strain, are morphologically similar to human lung adenocarcinomas, and furthermore, many of the signaling pathways with genetic and epigenetic alterations in oncogenes and tumor suppressor genes are identical to those that are found in human lung cancer37,39-41. Molecular aspects of lung cancer in humans and mice have also been well analyzed. K-ras mutations in lung cancer are well documented. K-ras mutations in human lung adenocarcinoma occur primarily at codon 1232, and its mutations in B6C3F1 mice were also found at Codon 12 in spontaneous lung tumors45. These findings show that the mouse lung carcinogenesis model is very suitable tool for identifying human lung carcinogens, promoters or modifiers, and chemopreventive agents of lung carcinogenesis.

Lung Cancer and CYP2A6

Cigarette smoke has been epidemiologically associated with lung cancer in humans. Kamataki et al. reported that Japanese male smokers with CYP2A6 gene deletion-type polymorphism were shown to have a reduced lung cancer risk in a hospital-based case control study44,45. Furthermore, CYP2A6 gene deletion reduced oral cancer risk in betel quid chewers in Sri Lanka46. 4-(MethylNitrosamino)-1-(3-pyridyl)-1-butanone (NNK), one of tobacco-specific N-nitrosamines, plays an important role in tobacco-related human lung cancer, and it has strong potential to induce lung tumorigenesis in rodents47. If one of the causes of human lung cancer is dependent on metabolic activation of a tobacco-specific nitrosamine, inhibition of CYP2A6 by chemicals may result in chemoprevention of tobacco-related lung cancer. Therefore, we examined the potential inhibitory effects of 8-methoxypsorarene (8-MOP), a potent human CYP2A6 inhibitor, on NNK-induced lung tumorigenesis in female A/J mice48.

A/J female mice were pretreated with 8-MOP (50 or 12.5 mg/kg body weight in 0.2 ml corn oil, i.g.) or equal volume of corn oil (vehicle control) daily for 3 days. One
hour after the final treatment, animals were given a single dose of NNK (2 mg/0.1 ml saline/mouse, i.p.) or equal volume of saline. They were then maintained without additional treatment, and the experiment was terminated 16 weeks after the first 8-MOP treatment. At week 16 of the experiment, the animals were sacrificed and lung lesions were analyzed. All of the macroscopically detected lung nodules were counted and each lung lobe was examined histopathologically. Lung proliferative lesions, alveolar cell hyperplasia, adenoma and adenocarcinoma were diagnosed according to the criteria of "Tumors of the mouse"49, and the number of lesions were counted under a microscope.

Incidences and tumors/mouse of NNK-induced lung adenomas in mice treated with 8-MOP are summarized in Table 1. Pretreatment of 8-MOP significantly reduced tumor incidence from 93.8% to 16.7% (50 mg/kg body weight) and 20.0% (12.5 mg/kg body weight), and tumor multiplicity from 5.97 to 0.23 (50 mg/kg body weight) and 0.25 (12.5 mg/kg body weight) tumors/mouse. These results indicate that 8-MOP, a potent human CYP2A6 inhibitor, is a strong chemopreventive agent for NNK-induced A/J mouse lung tumorigenesis48.

Dose dependent inhibitory effects of dietary 8-MOP on NNK-induced lung tumorigenesis were also examined50. Female A/J mice were fed diets supplemented with 8-MOP at concentrations of 100, 10 or 1 ppm in their basal diets for 3 days. Three days after the first treatment, each group was given a single dose of NNK (2 mg/0.1 ml saline/mouse, i.p.) or an equal volume of saline (vehicle control). Control groups were treated with NNK alone and 100 ppm 8-MOP + saline. They were then fed basal diets and maintained without further treatment until the termination after 16 weeks. At autopsy, their lungs were excised and weighed, and all macroscopically detected lung nodules were counted under a stereomicroscope. In addition, each lung lobe was examined histopathologically. mRNA levels of CYP2A4 and CYP2A5 were also analyzed using the reverse transcriptase-polymerase chain reaction. Mouse CYP2A4 and CYP2A5 differ from each other by only 11 amino acids, and these enzymes are closely related to human CYP2A651. Incidences and multiplicities of NNK-induced lung hyperplasias and adenomas in mice treated with different

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### Table 1. Incidences and Tumors/Mouse of NNK-Induced Lung Adenoma in A/J Mice Treated with 8-Methoxypsoralen (8-MOP)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 1 and Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Incidence (%)</td>
<td>Tumors/mouse</td>
<td>No. Incidence (%)</td>
<td>Tumors/mouse</td>
</tr>
<tr>
<td>1a</td>
<td>8-MOP (50 mg)+NNK</td>
<td>10</td>
<td>0/10 (0)</td>
<td>20</td>
</tr>
<tr>
<td>1b</td>
<td>8-MOP (12.5 mg)+NNK</td>
<td>ND</td>
<td>1/11 (9.1)</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>8-MOP (50 mg)</td>
<td>11</td>
<td>0/11 (0)</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>NNK</td>
<td>15</td>
<td>14/15 (93.3)</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>Vehicle control</td>
<td>ND</td>
<td>14/15 (93.3)</td>
<td>14</td>
</tr>
</tbody>
</table>

a: Number of mice examined.
b: Number of mice observed with lesions (%).
c: Mean ± SD.
d: Significantly different from group 3 by Fischer’s exact probability test (P < 0.001).
e: Significantly different from group 3 by Student’s t test (P < 0.001).
f: ND, not determined.

(Modified from Takeuchi et al., 2003)

### Table 2. Incidences and Tumors/Mouse of NNK-Induced Lung Adenoma in A/J Mice Treated with 3 Different Dietary Doses of 8-Methoxypsoralen (8-MOP)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Hyperplasia</th>
<th>Adenoma</th>
<th>Hyperplasia+adenoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Incidence (%)</td>
<td>Tumors/mouse</td>
<td>No. Incidence (%)</td>
<td>Tumors/mouse</td>
</tr>
<tr>
<td>1</td>
<td>8-MOP (100 ppm)+NNK</td>
<td>20</td>
<td>5/20 (25.0)</td>
<td>10/20 (50.0)</td>
</tr>
<tr>
<td>2</td>
<td>8-MOP (10 ppm)+NNK</td>
<td>20</td>
<td>16/20 (80.0)</td>
<td>14/20 (70.0)</td>
</tr>
<tr>
<td>3</td>
<td>8-MOP (1 ppm)+NNK</td>
<td>20</td>
<td>15/20 (75.0)</td>
<td>17/20 (85.0)</td>
</tr>
<tr>
<td>4</td>
<td>NNK</td>
<td>20</td>
<td>19/20 (95.0)</td>
<td>15/20 (75.0)</td>
</tr>
<tr>
<td>5</td>
<td>8-MOP (100 ppm)+saline</td>
<td>20</td>
<td>2/20 (10.0)</td>
<td>1/20 (5.0)</td>
</tr>
</tbody>
</table>

a: Number of mice examined.
b: Number of mice observed with lesions (%).
c: Mean ± SD.
d: Significantly different from group 4 by Fischer’s exact probability test (P < 0.01).
e: Significantly different from group 4 by Student’s t test (P < 0.0001).
f: Significantly different from group 3 by Fischer’s exact probability test (P < 0.05).
g: Significantly different from group 4 by Student’s t test (P < 0.001).

(Modified from Takeuchi et al., 2006)
tumorigenesis have also been reported. Female A/J mice were pretreated with 8-MOP at a dose of 0.125, 1.25 or 12.5 mg/kg body weight in 0.2 ml saline, i.p. One hour after the last treatment, animals were given a single dose of NNK at a dose of 100 mg/kg body weight in 0.1 ml saline, i.p., or an equal volume of saline as a vehicle control. In separate experiments, 3 daily doses of 8-MOP (12.5 mg/kg body weight) were given to mice 1, 3 and 7 days after the single dose NNK injection. These experiments were terminated at week 16. At autopsy, lungs were excised and all of the macroscopically detected lung nodules were counted, and each lung lobe was analyzed histopathologically. Additionally, lungs were immunostained for CYP2A by the ABC method. Pretreatment of mice with 8-MOP inhibited the incidence and multiplicities of NNK-induced lung proliferative lesions in a dose dependent manner. The relative quantifications of CYP2A4 and CYP2A5 mRNAs in livers and lungs of A/J mice were not influenced by the 8-MOP treatment.

The mechanisms and timing of the 8-MOP chemoprevention of NNK-induced mouse lung tumorigenesis have also been reported. Female A/J mice were pretreated with 8-MOP at a dose of 0.125, 1.25 or 12.5 mg/kg body weight in 0.2 ml corn oil, or an equal volume of corn oil as a vehicle control. One hour after the last treatment, animals were given a single dose of NNK at a dose of 100 mg/kg body weight in 0.1 ml saline, i.p., or an equal volume of saline as a vehicle control. In separate experiments, 3 daily doses of 8-MOP (12.5 mg/kg body weight) were given to mice 1, 3 and 7 days after the single dose NNK injection. These experiments were terminated at week 16. At autopsy, lungs were excised and all of the macroscopically detected lung nodules were counted, and each lung lobe was analyzed histopathologically. Additionally, lungs were immunostained for CYP2A by the ABC method. Pretreatment of mice with 8-MOP inhibited the incidence and multiplicities of macroscopically and microscopically examined lung lesions. However, treatment of 8-MOP on days 1, 3 and 7 after NNK administration did not affect the incidence and multiplicities of the observed lung lesions. These results suggest that 8-MOP abolished NNK-induced lung tumorigenesis via the inhibition of an initiation event in lung carcinogenesis, but not subsequent events including promotion of carcinogenesis. In this experiment, the expression of mRNA for CYP2A5, but not for CYP2A4 or CYP2A12, in mouse lung was proved by reverse transcriptase-polymerase chain reaction, probably indicating that CYP2A5 present in the mouse lung was involved in the metabolic activation of NNK. Interestingly, tumor cells in lung adenoma were positive for CYP2A immunohistochemistry.

### Analysis of Chemopreventive Agents of Lung Carcinogenesis

Since lung cancer is one of the most common causes of mortality and morbidity in the world, new therapeutic strategies such as chemoprevention are a high priority. Lactoferrin (LF) is a multifunctional, iron-binding glycoprotein present mainly in external secretions, such as breast milk, tears, saliva and seminal fluid, and in the secondary granules of neutrophils. It was originally identified as a mucosal host defense mediator and anti-inflammatory modulator. Furthermore, LF has shown anti-tumor effects in vitro and in vivo in the colon, tongue, esophagus, urinary bladder, and possibly in the lung. We investigated the effects of orally administered LF using a mouse model with NNK-induced lung tumors, which histopathologically resemble human lung adenocarcinomas. Female A/J mice were divided into 5 groups, and they were administered 0.02, 0.2 and 2% bovine LF (bLF) in the powdered basal diet during the initial 1-week period. Animals were given a single dose of NNK (2 mg/0.1 ml saline/mouse, i.p.). Control animals received only NNK or 0.2% bLF alone. They were then maintained without additional treatment, and the experiment was terminated at week 16. Incidences and multiplicities of lung proliferative lesions, including alveolar cell hyperplasia, adenoma and adenocarcinoma were compared between groups. In further experiments, mice were administered 2% bLF throughout the experimental period. Another group was administered bLF from week 2 onwards. At week 1, mice were given a single dose of NNK (2 mg/0.1 ml saline/mouse, i.p.). Control groups were given NNK alone or bLF alone. For the short bLF treatment experiment, lung lesions were not significantly different. For the longer bLF treatment, macroscopically counted lung nodules were significantly reduced by orally administered 2% bLF during the post-initiation phase. There were no significant differences in incidences of macroscopically diagnosed lung proliferative

### Table 3. PCNA and Cleaved Caspase-3-Labeling Indices of Hyperplasias and Adenomas in A/J Female Mice Treated with NNK and Bovine Lactoferrin (bLF)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>PCNA-labeling index ratio</th>
<th>Cleaved caspase-3-labeling index ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. a Hyperplasia</td>
<td>No. a Adenoma</td>
</tr>
<tr>
<td>1</td>
<td>bLF (whole) +NNK</td>
<td>9  3.81 ± 0.51 b</td>
<td>11 10.00 ± 1.16</td>
</tr>
<tr>
<td>2</td>
<td>bLF (post-initiation)+NNK</td>
<td>4 1.53 ± 0.29</td>
<td>9 6.91 ± 1.07 c</td>
</tr>
<tr>
<td>3</td>
<td>NNK</td>
<td>8 4.10 ± 0.60 d</td>
<td>11 11.76 ± 1.13 d</td>
</tr>
<tr>
<td>4</td>
<td>bLF (whole)+saline</td>
<td>ND d ND d</td>
<td>ND ND d</td>
</tr>
</tbody>
</table>

(Modified from Matsuda et al., 2007)

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a: Effective number of lesions which were included in immunohistochemical analyses. 
b: Mean ± SD. 
c: Significantly different from groups 1 and 3 by the post hoc test (P < 0.05). 
d: ND; not determined.
nODULES. Immunohistochemical data of PCNA, a cell proliferation marker, and cleaved caspase-3, an apoptosis marker, are shown in Table 3. The mean PCNA-labeling index ratios in hyperplasias and adenomas of bLF during the post-initiation phase were lower than those of the NNK alone group. The mean cleaved caspase-3-labeling index ratios in adenomas showed a tendency to increase in the bLF treated groups compared to the NNK alone group, but without statistical significance. These data showed chemopreventive effects of dietary bLF on NNK-induced lung tumorigenesis in mice, through modification of cell proliferation and/or apoptosis.

Conclusion

Detection of carcinogenic or tumor promoting substances of lung carcinogenesis is very important in order to reduce potentially harmful chemicals in our environment. Therefore, detection of chemopreventive agents of lung carcinogenesis is important for reducing the risk of lung cancer. For that purpose, it is necessary to establish reliable in vivo animal models of lung carcinogenesis.

The A/J mouse is sensitive to lung carcinogens and spontaneous lung tumors also develop without any chemical treatment. NNK, a tobacco specific nitrosamine, and MeQx, a heterocyclic amine, induced lung tumors in 16 and 32 weeks in female A/J mice. The lung tumors developed in the A/J mouse were histopathologically classified as adenocarcinomas, adenomas, and alveolar cell hyperplasias. Using the A/J mouse, we reported that 8-MOP, a potent human CYP2A6 inhibitor, is a strong chemopreventive agent of NNK-induced lung tumorigenesis.

We also investigated the effects of bLF on different phases of NNK-induced lung tumorigenesis in A/J mice. bLF administered during the post-initiation phase caused a significant reduction in macroscopical lung nodules. bLF might inhibit NNK-induced mouse lung tumorigenesis, only when given in the post-initiation phase, through modification of cell proliferation and/or apoptosis.

The A/J mouse is very useful mouse strain as a reliable in vivo model of lung carcinogenesis, which can be used for determining chemopreventive agents of lung carcinogenesis.

References


