Lack of Modifying Effects of Intratracheal Instillation of Quartz or Dextran Sulfate Sodium (DSS) in Drinking Water on Lung Tumor Development Initiated with 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in Female A/J Mice

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Abstract: The purpose of the present study was to investigate the effects of inflammation, induced by intratracheal instillation (i.t.) of quartz as an environmental factor in the lung or drinking of dextran sulfate sodium (DSS) as an environmental factor in the colon on lung tumors in female A/J mice initiated with NNK. For comparison, colonic preneoplastic lesions, aberrant crypt foci (ACF), were also assessed. A/J mice at 6 weeks of age were divided into 5 groups, and Groups 1, 2 and 3 were pretreated with NNK (2 mg / 0.1 ml saline / mouse, intraperitoneal injection) at week 0. For a week, 2% DSS in drinking water was administered to the mice in Groups 2 and 4 beginning in week 1. In week 2, the mice of Groups 3 and 5 were exposed to intratracheal instillation of quartz (0.1 mg/rat) suspended in 25 μl saline. The experiment was terminated after 16 weeks. The results for the lung tumors and colonic ACFs showed a lack of modifying effects of the inflammation in either site. Hematologically and histopathologically, the inflammation induced by 0.1 mg quartz in the lung and 2% DSS in the colon was lacking or only mild at the end of 16 weeks. These results suggest that there may be differences in sensitivity to inflammation that determine tumor promoting potential.

Key words: NNK, lung tumor, quartz, DSS, inflammation, A/J mouse

Introduction

Lung cancer is common throughout the world and a leading cause of mortality in developed countries. While cigarette smoking is regarded as the primary cause, with cessation of smoking being the major target for reduction of incidence, the risk of development of lung cancer development remains elevated even after individuals quit smoking and exposure to environmental tobacco smoke from others continues to be a problem. Therefore, it is important to investigate the biological characteristics of lung tumors and the effects of environmental factors on lung tumorigenesis.

The tabacco-specific N-nitrosamine, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), conceivably plays an important role in tobacco-related human lung cancer given its strong potential to induce lung lesions in rodents. However there are no reports concerning NNK and colonic tumors or colonic preneoplastic lesions, aberrant crypt foci (ACF). Previously, we demonstrated that treatment with 8-methoxypsoralen (8-MOP) during the initiation phase strongly inhibits the lung tumorigenesis induced by a single intraperitoneal (i.p.) injection of NNK in female A/J mice. CYP2A6 is recognized as being involved in the mutagenic activation of promutagens such as tobacco-specific N-nitrosamines and is also responsible for metabolism of 70%–80% of nicotine to the inactive metabolite cotinine in humans. It has also been reported that 8-MOP inhibits CYP2A6 and we have previously demonstrated that 3-day intake of 100 ppm 8-MOP strongly reduces induction of lung tumors. When adenocarcinomas induced by single i.p. injection of NNK in A/J mice for 52 weeks were used for a mutation analysis, high rates of change were noted in codons 12 and/or 61 of K-ras (positive in 21/23 lung tumors). We have also focused on establishing a short-term bioassay...

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model for identification of chemopreventive agents acting during the initiation phase of lung carcinogenesis. Examination of the time course of NNK-induced lung tumor development to determine the most appropriate and shortest period to assess the effects of test agents, with 8-MOP as a typical example, showed that two treatments with NNK and a 12-weeks duration are effective for detection of lung cancer chemoprevention.

There are many reports showing that inflammation promotes carcinogenic potential in various organs. The mechanism of the promotion has also been reported to induce cytokines, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), NF-kappa B, tumor growth factor (TNF) and so on by inflammatory changes. If their markers are transported by the blood flow from the primary organ of the inflammation to other organs, the promotive potential may be induced in the organs that are distant from the original site of inflammation.

The purpose of the present study was to investigate the effects of inflammation, induced by intratracheal instillation (i.t.) of quartz as an environmental factor in the lung or drinking of dextran sulfate sodium (DSS) as an environmental factor in the colon, on lung tumors in female A/J mice initiated with NNK. For comparison, colonic preneoplastic lesions, aberrant crypt foci (ACF), were also assessed. Quartz and DSS are typical agents causing inflammation in the lung and the colon, respectively. To assess the effect of the inflammation in the local organs, which were also the carcinogen-initiated organs, assessments of the effects of lung inflammation on lung tumor and colonic inflammation on ACF in the colon were performed. Furthermore, to assess the effect of the inflammation in the distant organs, which were not carcinogen-initiated organs, assessments of the effects of lung inflammation by quartz on ACF in the colon and colonic inflammation by DSS on lung tumor were also performed.

Materials and Methods

Chemicals
NNK was purchased from Toronto Research Chemicals (Toronto, ON, Canada), and DSS (molecular weight: 36000–50000 daltons) was purchased from MP Biomedicals Inc. (Burlingame, CA, USA); quartz dust (DQ-12) with a particle diameter of not more than 7 μm was obtained from Deutsche Montan Technologie (GmbH, Germany). Physiological saline (Otsuka Isotonic sodium chloride solution from Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) was used as the vehicle for both test substances.

Animals
Female A/J mice (5 weeks of age), purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan), were maintained in the Division of Animal Experimentation, Life Science Research Center, Kagawa University, according to the Institutional Regulations for Animal Care and Use Committee for Kagawa University. The animals were housed in polycarbonate cages with white wood chips for bedding, and given free access to drinking water and a basal diet, Oriental MF (Oriental Yeast Co., Ltd., Tokyo, Japan), under controlled conditions of humidity (60 ± 10 %), lighting (12 h light/dark cycle) and temperature (24 ± 2°C). The experiments were started after a 1-week acclimation period.

Experimental design and tissue preparation
The design for the experiment is outlined in Fig. 1. A total of 80 mice at 6 weeks of age were divided into 5 groups of 15 (Groups 1, 2 and 5), 30 (Group 3) and 5 mice (Group 4). The mice of Groups 1, 2 and 3 were pretreated with NNK (A: 2 mg / 0.1 ml saline / mouse i.p.) in week 0. The mice of Groups 2 and 4 received 2% DSS (■) in drinking water for one week from week 1. In weeks 2, the mice of Groups 3 and 5 were exposed to intratracheal instillation of quartz (△: 0.1 mg/rat) suspended in 25 ml saline. The experiment was terminated after 16 weeks (S: sacrifice).

![Experimental design](image)

Fig. 1. Experimental design. A total of 80 A/J mice, were divided into 5 groups at 6 weeks of age. Groups 1, 2 and 3 were pretreated with NNK (A: 2 mg / 0.1 ml saline / mouse i.p.) in week 0. The mice of Groups 2 and 4 received 2% DSS (■) in drinking water for one week from week 1. In weeks 2, the mice of Groups 3 and 5 were exposed to intratracheal instillation of quartz (△: 0.1 mg/rat) suspended in 25 ml saline. The experiment was terminated after 16 weeks (S: sacrifice).
routinely processed for embedding in paraffin for histopathological examination of hematoxylin and eosin-stained sections and immunohistochemistry. In the hematological examinations, the red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT) and white blood cell count (WBC) were determined using routine laboratory procedures; the hematological examinations were performed by SRL, Inc. (Tokyo, Japan).

### Statistical analysis

The incidences of ACFs in the colons were analyzed by the Kruskal-Walllis test. The body and organ weight data, hematological data, and the multiplicities of lung proliferative lesions and ACFs in the colon were analyzed by Tukey-Kramer test (multi-comparison test).

### Results

A total of 10 mice died during the experimental period, seven (6 mice of Group 3 and 1 of Group 5) after intratracheal instillation of quartz, and the other 3 (2 mice of Group 1 and 1 of Group 2) spontaneously during the experimental period. None of the groups demonstrated any remarkable changes in their general conditions.

The final body and organs weights are shown in Table 1. The body weights of the mice in Groups 2 and 3 were significantly decreased compared with those of the mice in Groups 4 and 5, respectively. The absolute weights of the liver and left kidneys of the mice in Groups 2 and 3 were also decreased compared with the mice in Groups 4 and 5, respectively, but the relative weights did not show any significant differences.

For the hematological studies, 9–17 mice per group were examined due to technical errors in blood collection. The WBCs of the mice in Group 3 (NNK+quartz) were significantly decreased compared with the Group 4 values (DSS). However, no significant intergroup differences were noted in Hb (shown in Table 2), RBC, HT, MCV, MCH and MCHC (data not shown).

Macroscopically, whitish nodules were detected in the lungs of the NNK-treated mice in Groups 1, 2 and 3; their multiplicities based on counting under a stereomicroscope are summarized in Table 3. Contrary to expectation, there were no significant intergroup differences. No significant macroscopic changes were observed in the liver, kidney or colon. ACFs were seen in some mice treated with NNK.

### Table 1. Body and Organ Weights of the Mice

<table>
<thead>
<tr>
<th>Groups Treatment</th>
<th>1 NNK i.p.</th>
<th>2 NNK i.p.+DSS</th>
<th>3 NNK i.p.+quartz i.t.</th>
<th>4 DSS</th>
<th>5 Quartz i.t.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>13</td>
<td>14</td>
<td>24</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>24.50 ± 1.67</td>
<td>22.83 ± 1.78b</td>
<td>22.79 ± 1.97c</td>
<td>26.53 ± 3.14</td>
<td>28.22 ± 3.43</td>
</tr>
<tr>
<td>Liver Absolute (mg)</td>
<td>1089 ± 56</td>
<td>954 ± 77b</td>
<td>993 ± 96c</td>
<td>1127 ± 96</td>
<td>1127 ± 199</td>
</tr>
<tr>
<td></td>
<td>4.46 ± 0.26</td>
<td>4.18 ± 0.21</td>
<td>4.36 ± 0.29</td>
<td>4.27 ± 0.30</td>
<td>3.99 ± 0.43</td>
</tr>
<tr>
<td>Kidneys Right Absolute (mg)</td>
<td>211 ± 316</td>
<td>113 ± 10</td>
<td>118 ± 12</td>
<td>143 ± 9</td>
<td>139 ± 23</td>
</tr>
<tr>
<td>Relative (%)</td>
<td>0.86 ± 1.27</td>
<td>0.49 ± 0.03</td>
<td>0.52 ± 0.04</td>
<td>0.54 ± 0.05</td>
<td>0.49 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>125 ± 10</td>
<td>111 ± 9b</td>
<td>119 ± 11c</td>
<td>143 ± 14</td>
<td>133 ± 21</td>
</tr>
<tr>
<td>Left Absolute (mg)</td>
<td>0.51 ± 0.04</td>
<td>0.49 ± 0.01</td>
<td>0.52 ± 0.04c</td>
<td>0.54 ± 0.04</td>
<td>0.47 ± 0.06</td>
</tr>
<tr>
<td>Relative (%)</td>
<td>166 ± 12</td>
<td>171 ± 18</td>
<td>170 ± 20</td>
<td>177 ± 13</td>
<td>175 ± 25</td>
</tr>
<tr>
<td></td>
<td>0.68 ± 0.07</td>
<td>0.75 ± 0.09</td>
<td>0.75 ± 0.06</td>
<td>0.67 ± 0.07</td>
<td>0.63 ± 0.10</td>
</tr>
</tbody>
</table>

*a P<0.05 vs group 1 by Tukey-Kramer test. b P<0.05 vs group 4. c P<0.05 vs group 5.

### Table 2. Hematological Data of the Mice

<table>
<thead>
<tr>
<th>Groups Treatment</th>
<th>1 NNK</th>
<th>2 NNK+DSS</th>
<th>3 NNK+quartz</th>
<th>4 DSS</th>
<th>5 Quartz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice</td>
<td>6</td>
<td>13</td>
<td>17</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>WBC</td>
<td>2583 ± 1037</td>
<td>2215 ± 585</td>
<td>1635 ± 956*</td>
<td>2900 ± 1109</td>
<td>2445 ± 770</td>
</tr>
<tr>
<td>Hb</td>
<td>14.0 ± 0.3</td>
<td>14.5 ± 0.8</td>
<td>14.6 ± 0.5</td>
<td>15.1 ± 0.7</td>
<td>14.8 ± 0.7</td>
</tr>
</tbody>
</table>

*a P<0.05 vs. Group 4 by Tukey-Kramer test.
Lack of Modifying Effects of Inflammation

There were no significant intergroup differences in these values or the number of crypts per ACF.

Histopathologically, evidence of inflammation in the lungs was only slight with intratracheal instillation of quartz in Groups 3 (Fig. 2-B) and 5 as compared with the NNK alone group, Group 1 (Fig. 2-A). DSS also induced only mild inflammatory changes in the colons of the mice in Group 2 (Fig. 2-D) and 4 compared with the NNK alone group, Group 1 (Fig. 2-C).

Discussion

In the present experiment, there were no significant modifying effects of the inflammation-inducing regimens in distant or local target organs of NNK carcinogenesis in the female A/J mice. Thus, the quantitative data for the lung tumors and colon ACFs showed no changes. Furthermore, hematologically and histopathologically, the inflammation induced by 0.1 mg quartz in the lung and 2% DSS in the colon was milder than expected. This suggests that female A/J mice may be relatively insensitive to these agents.

In quartz dust-exposed construction workers, obstructive and restrictive loss of lung function has been detected, as well as chronic obstructive pulmonary disease (COPD). These conditions are associated with an inflammatory cell response characterized by alveolitis with recruitment of inflammatory cells, particularly neutrophils, and may result in pulmonary fibrosis and impaired lung function. Intratracheal instillation of quartz into rats produces an inflammatory reaction followed by histological changes characteristic of lung fibrosis, which is similar to the above-noted human conditions. We have also employed quartz as a positive control for material induced lung inflammation in F344 rats and have established a bioassay model by intratracheal instillation for detection of lung toxicity due to fine particles in F344 male rats. From these previous experiments in our laboratory, 0.1 mg quartz was concluded to be a sufficient dose to cause inflammation in the lungs of a mouse (estimated average body weight: 20 g) based on our previous findings for 1 mg quartz in F344 rats (estimated average body weight: 200 g).

DSS is a synthetic sulfated polysaccharide well known to induce inflammatory changes in the colons of mice, rats, hamsters, and guinea pigs. It has been reported that BALB/c mice develop acute colitis with signs of diarrhea, gross rectal bleeding, and weight loss within 6–10 days after ingesting 3%–10% DSS. The most widely used inflammatory bowel disease model is that of mice given DSS, and Tanaka et al. reported that 1-week administration of 2% DSS after initiation with azoxymethane (AOM, 10 mg/kg body weight i.p.) in male ICR mice, or a single i.g. administration (200 mg/kg body weight) of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) or 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) in CD-1 mice, resulted in powerful promoting activity on colon carcinogenesis. This was associated with induction of betacatenin, cyclooxygenase-2 and inducible nitric oxide synthase. In the present experiment, however, the dose of 2% DSS administration had no without major influence. This is in line with one report of mouse strain differences in the inflammatory responses of the colonic mucosa induced by dextran sulfate sodium and thus differential susceptibility to PhIP-induced large bowel carcinogenesis.

From the dose information of other reports and our previous experiments, both 0.1 mg quartz i.t. and

| Table 3. Macroscopical Nodules in the Lungs |
|-------------------|-----|-----|-----|
| Groups | Treatment | No. | Right | Left | Bilateral |
| 1 | NNK | 13 | 2.2 ± 2.1 | 1.2 ± 1.8 | 3.4 ± 3.6 |
| 2 | NNK+DSS | 14 | 2.4 ± 1.7 | 1.4 ± 1.5 | 3.8 ± 2.8 |
| 3 | NNK+quartz | 24 | 1.8 ± 1.5 | 1.4 ± 1.6 | 3.2 ± 2.3 |
| 4 | DSS | 5 | 0.2 ± 0.4 | 0.0 | 0.2 ± 0.4 |
| 5 | Quartz | 14 | 0.0 | 0.2 ± 0.4 | 0.2 ± 0.4 |

There was no significant intergroup difference by Tukey-Kramer test.

| Table 4. ACFs Analysis in the Colon |
|-----------------|-----|-----|-----|
| Groups | No. | Incidence (%) | No. of ACFs | No. of crypts |
| 1 | NNK | 13 | 38.5 | 0.62 ± 0.96 | 4.50 ± 2.78 |
| 2 | NNK+DSS | 14 | 35.7 | 0.50 ± 0.76 | 6.86 ± 2.34 |
| 3 | NNK+quartz | 24 | 20.8 | 0.25 ± 0.53 | 7.00 ± 2.45 |
| 4 | DSS | 5 | 0.0 | 0.00 | 0.00 |

Incidence: there was no significant intergroup difference by Kruskal-Wallis test. ACFs and crypts: there was no significant intergroup difference by Tukey-Kramer test.
administration of 2% DSS in drinking water were expected to induce inflammatory changes in each target organ in the present experiment, however, the results were contrary to our expectation. The results of our present experiment indicate that the A/J mouse may have high resistibility against treatments including an inflammatory reaction. Furthermore, there may be specific differences in inflammatory responses to quartz and DSS. An experiment is currently being performed to confirm this hypothesis.

In conclusion, the results of the present experiment showed a lack of modifying effects of inflammation-targeting regimens on the development of lung tumors and colonic ACFs initiated with NNK in female A/J mice indicative of species or strain differences in sensitivity to the agents employed. However the general tumor promoting effects of inflammation, assessment of alternative inducing regimens is warranted.

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References


