Characterization of Early Pulmonary Hyperproliferation and Tumor Progression and Their Inhibition by Black Tea in a 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced Lung Tumorigenesis Model with A/J Mice

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ABSTRACT

The pathogenesis of pulmonary tumors induced by a tobacco carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and its inhibition by black tea have been characterized. Female A/J mice (6 weeks old) were treated with a single dose of NNK (103 mg/kg of body weight, ip.) on day 0, and the cell proliferation index was measured by the incorporation of bromodeoxyuridine (BrdUrd) immunohistochemically. The number of BrdUrd-labeled cells increased in the bronchiolar epithelium from day 2 to day 14, with the highest proliferation rate observed on day 5. By day 35, the BrdUrd-labeling index returned to the level of the control group. Further examination of the day 35 samples revealed the presence of foci of hyperproliferative cells in the bronchiolar epithelium, particularly in the bronchiolalveolar regions. These proliferating bronchiolar epithelial cells (Clara cells) may be the initiated sites for pulmonary tumorigenesis. In this short-term model, administration of black tea polyphenols (0.3%) through the drinking water starting 24 h after NNK treatment significantly inhibited NNK-induced early bronchiolar cell proliferation on day 5. In long-term studies, adenomas were observed in 100% (15 of 15) of the mice at week 16, with 7.8 ± 0.8 tumors per mouse. At week 52, a malignant tumor incidence of 80% (41 of 51 mice) and a malignant tumor multiplicity of 2.39 ± 0.19 were observed. The growth patterns of the malignant tumors, which included solid, papillary, and mixed types, may be associated with the cellular origin of the tumor. The cell proliferation indices, as measured by proliferating cell nuclear antigen immunohistochemistry, were significantly higher in dysplasia within adenoma than in adenoma, and significantly higher in adenoma at week 52 than in adenoma at week 16. Administration of black tea, starting 16 weeks after a single dose of NNK, inhibited the progression of adenoma to adenocarcinoma as determined by both malignant tumor incidence and multiplicity. The cell proliferation rate in adenomas was also suppressed by black tea treatment. The present work demonstrates the antiproliferative activities of black tea and its polyphenols. Such activities, at the early and late stages of lung tumorigenesis, may be important for the cancer-chemopreventive activities of black tea.

INTRODUCTION

Lung cancer is one of the most common cancers worldwide and has the highest mortality rate among malignant tumors in the United States (1). More than 85% of lung cancers may be attributed directly to cigarette smoking (2). NNK4 is a potent tobacco carcinogen, and its carcinogenicity has been demonstrated in many animal models (17, 18). Previous studies have demonstrated that black and green tea inhibited NNK-induced pulmonary adenomas in mice (19–21). The inhibitory effect was observed even when tea was administered (in drinking water) starting 1 or 5 weeks after NNK treatment (19, 20), but the mechanisms of action are not known. Tea is one of the few agents known to inhibit carcinogenesis at the postinitiation stage (19–21). It would be interesting to determine whether tea inhibits the progression of benign to malignant tumors. Tea catechins, especially EGCG, are considered to be the active components by many investigators, but the quantities of catechins are reduced greatly during the manufacturing of black tea. The characteristic black tea polyphenols are theaflavins and the poorly characterized “thearubigns.” Whether theaflavins or other black tea polyphenols can inhibit NNK-induced early pulmonary proliferation and the progression of adenoma to adenocarcinoma is not known.

The purpose of the present study was (a) to investigate the early pulmonary cell proliferation in A/J mice after treatment with NNK, (b) to characterize the morphological progression of adenoma to adenocarcinoma, and (c) to examine the inhibitory effects of black tea and black tea polyphenols against cell proliferation and tumor progression.
MATERIALS AND METHODS

Materials. NNK was obtained from Chemsyn Science Laboratories (Lenexa, KS). Powdered forms of "black tea solids," "black tea polyphenols," and "theaflavins" were provided by the Thomas J. Lipton Tea Company (Englewood Cliffs, N.J.). The black tea solids were dehydrated water extracts of a blend black tea. One g of the black tea solids contained approximately 15 mg of theaflavins, 70 mg catechins (of which 30 mg were EGCG), 340 mg of undefined polyphenols (mainly thearubigins), and 50 mg of caffeine. The black tea polyphenols were prepared by extracting black tea water extracts with ethyl acetate, and the ethyl acetate fraction was decaffeinated by methylene chloride and then freeze-dried. One g of black tea polyphenols contained 130 mg theaflavins, 175 mg EGCG, 106 mg (−)-epicatechin-3-gallate, 56 mg (−)-epicatechin, and 16 mg (−)-epigallocatechin. The theaflavins were a mixture of theaflavin (21%), theaflavin-3-gallate (30%), theaflavin-3'-gallate (15%), and theaflavin-3,3'-digallate (28%). The AIN-76A diet was purchased from Teklad Co. (Madison, WI). BrdUrd was purchased from Sigma Chemical Co. (St. Louis, MO).

Treatment of Animals. Female A/J mice (6 weeks old, from The Jackson Laboratory, Bar Harbor, ME) were treated with a single dose of NNK (103 mg/kg, i.p.) or saline. The animals were fed an AIN-76A diet and given water ad libitum. They were maintained in air-conditioned quarters with a room temperature of 20 ± 2°C, relative humidity of 50 ± 10%, and an alternating 12-h light/dark cycle.

The purpose of the first experiment was to study early pulmonary proliferation as a response to NNK treatment. The mice were sacrificed on days 1, 2, 3, 5, 7, 14, and 35 (five mice per time point for the NNK treatment groups and three mice per time point for the saline control groups). BrdUrd was dissolved in sterile normal saline and administered i.p. to the mice 2 h before sacrifice at 50 μg/g of body weight. The lungs were removed, inflated, fixed in Carnoy's solution, and embedded in paraffin.

In the second experiment, the effects of black tea polyphenols and theaflavins on NNK-induced early pulmonary proliferation were studied. Solutions of black tea polyphenols (0.3%) and theaflavins (0.1%) were made fresh in warm, deionized water daily and administered to the mice as the sole source of drinking fluid 24 h after NNK treatment until the termination of the experiment. Animals were sacrificed on day 5, and the BrdUrd-labeled pulmonary cell proliferation index was measured.

The purpose of the third experiment was to study the progression of NNK-induced adenoma to adenocarcinoma and its possible inhibition by black tea. At week 16, 15 mice from the NNK treatment group were sacrificed to confirm the expected pulmonary adenoma formation, and the remaining mice were given solutions of black tea solids (0.6%) as the sole source of drinking fluid until the termination of the experiment. The body weight, food intake, and water consumption were monitored biweekly. Because of the lower body weight of the mice found in the tea treatment group, the concentration of tea was changed to 0.3% starting at week 28. The experiment was terminated at week 52. The lungs were inflated and fixed with 10% buffered formalin. The proliferation index, i.e., the percentage of BrdUrd-positive cells of the bronchiolar or alveolar cells, was determined using a Nikon research microscope combined with Image-Pro Plus system (Media Cybernetics, Silver Spring, MD). For each slide, at least 10 bronchioli and 10 regions of alveoli were counted, and the total number of cells counted was more than 2000.

For the long-term (third) experiment, the proliferation index of the lungs in mice sacrificed at weeks 16 and 52 were examined immunohistochemically using the 2nd and 21st slides of each series of sections. The staining procedure was the same as above, except 10% buffered formalin and anti-PCNA antibody (1 μg/ml; Oncogene Science, Uniondale, NY) were used in place of Carnoy's fixative solution and anti-BrdUrd antibody, respectively. The percentage of PCNA-positive cells in the lesion region (proliferation index) was determined as above with the image system. More than three fields in each lesion were counted, and the total number of cells counted was more than 2000.

Statistical Analysis. The results on tumor incidence were analyzed by the χ² test, and the data on proliferation index and tumor multiplicity were analyzed by the Student's t test using the computer software Statview 4.2.

RESULTS

NNK-induced Early Pulmonary Hyperproliferation and Its Inhibition by Black Tea Polyphenols. In the first experiment, A/J mice were treated with NNK (on day 0) and sacrificed on days 1, 2, 3, 5, 7, 14, and 35. In the control group (Fig. 1a), the pulmonary cells labeled by BrdUrd in the alveolar and bronchiolar epithelia accounted for 0.51 and 0.46% of the cells, respectively. In the NNK-treated mice, no significant difference from the control mice was observed in the alveolar epithelial cells (proliferation indices, 0.36—0.76% from days 1—35). On the other hand, hyperproliferation of the bronchiolar

![Fig. 1. NNK-induced early pulmonary hyperproliferation (BrdUrd-immunostain and immunohistochemistry). a, lung from a control mouse (day 5): several BrdUrd-labeled cells (arrows) were observed in the bronchiolar epithelium (magnification, ×400); b, lung from a NNK-treated mouse (day 5): several BrdUrd-labeled labeled cells (arrows) were observed in the bronchiolar epithelium but not in the alveolar cells (magnification, ×400); c and d, lungs from NNK-treated mice (day 35): BrdUrd-labeled cell foci were found in the bronchiolar (c; magnification, ×400) and bronchiol-alveolar (d; magnification, ×450) regions.](image-url)
may have antiproliferative activities, but a higher concentration (than 0.1%) may be needed to display a significant effect.

**Proliferation and Progression of Lung Tumorigenesis.** In the third experiment, all 15 mice in the NNK-treated group sacrificed at week 16 had lung tumors, with a tumor multiplicity of 7.80 ± 0.80 per mouse. All of the tumors were monomorphic adenomas. At week 52, a tumor incidence of 100% and a tumor multiplicity of 8.78 ± 0.39 were found in a total of 51 mice. This included a malignant tumor incidence of 80% with a malignant tumor multiplicity of 2.39 ± 0.19.

Fig. 3 illustrates typical lesions of alveolar hyperplasia (Fig. 3a), adenoma (Fig. 3b), dysplasia within adenoma (Fig. 3c), adenocarcinoma within adenoma (Fig. 3d), and adenocarcinoma of solid growth (Fig. 3e) and papillary growth patterns (Fig. 3f). In a total of 118 malignant tumors, 17.8% (21 of 118) were adenocarcinomas with a solid growth pattern, 19.5% (23 of 118) were adenocarcinomas with a papillary growth pattern, and 62.7% (74 of 118) were adenocarcinomas within adenomas. In adenocarcinoma within adenoma, 91% were solid growth adenocarcinomas, and 9% were mixed adenocarcinomas, which exhibited both the solid and papillary growth patterns.

The numbers of lesions and the percentage of PCNA-labeled cells are summarized in Table 2. The proliferation index for adenomas at week 52 was 24.9 ± 0.57%. This was the weighted average of the monomorphic adenomas (23.2 ± 1.1%) and adenomas within adeno-

epithelia was observed in the NNK-treated mice sacrificed on days 2–14 and was rather prominent on day 5 (Fig. 1b). The proliferation index increased significantly from the controls on days 3, 5, and 7 and returned to the control level by day 35 (Fig. 2). Proliferative cell foci, defined as the aggregation of more than five BrdUrd-labeled cells (Fig. 1, c and d), were found in the bronchiolar epithelium of the NNK-treated mice on day 35, particularly in the bronchiolar-alveolar regions. By analyzing all of the serial sections from the whole lung, the average number of foci was determined to be approximately five per lung. Such foci were not found in the lungs of the control mice.

In the second experiment, 0.3% black tea polyphenols and 0.1% theaflavins were administered to the mice in their drinking fluid 24 h after NNK treatment. On day 5, the BrdUrd-labeled bronchiolar epithelial cells were counted. NNK-induced bronchiolar cell proliferation on day 5 was inhibited significantly by treatment with black tea polyphenols, but the antiproliferative effect of theaflavins was not statistically significant (Table 1). The mice in the theaflavin group received an estimated 2 mg of theaflavins per day, higher than the estimated theaflavin intake (0.87 mg per day) in the black tea polyphenol group. The results suggest that the theaflavins in the black tea polyphenol preparation cannot account fully for the antiproliferative activity and that other components possess such activity. Theaflavins

Table 1 Inhibitory action of black tea polyphenols against NNK-induced early bronchiolar epithelial cell proliferation

Mice were treated as described in "Materials and Methods" and were killed on day 5. Values are the mean ± SE of five mice for the NNK- and tea-treated groups and of three mice for the control group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proliferation index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.21 ± 0.07</td>
</tr>
<tr>
<td>NNK</td>
<td>4.63 ± 0.86</td>
</tr>
<tr>
<td>NNK + black tea polyphenols (0.3%)</td>
<td>1.84 ± 0.13</td>
</tr>
<tr>
<td>NNK + theaflavins (0.1%)</td>
<td>3.24 ± 0.42</td>
</tr>
</tbody>
</table>

*Significantly different from control (P < 0.001).

*Significantly different from the NNK group (P < 0.05).

Table 2 Pulmonary lesions and PCNA-labeling index in the A/J mice treated with NNK

A/J mice were sacrificed at week 52 after a single dose of NNK (103 mg/kg of body weight, i.p.). The pulmonary lesions were analyzed by histopathology and expressed as total lesion number and mean ± SE per mouse from 51 mice. PCNA-labeling indices in different lesioned areas are expressed as mean ± SE.

<table>
<thead>
<tr>
<th>Alveolar hyperplasia</th>
<th>Bronchiolar hyperplasia</th>
<th>Adenoma</th>
<th>Dysplasia within adenoma</th>
<th>Adenocarcinoma within adenoma</th>
<th>Adenocarcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>87</td>
<td>267</td>
<td>523 ± 0.37</td>
<td>1.15 ± 0.18</td>
<td>1.75 ± 0.24</td>
</tr>
<tr>
<td>Mean lesion No./mouse</td>
<td>0.33 ± 0.03</td>
<td>1.71 ± 0.30</td>
<td>5.23 ± 0.37</td>
<td>1.15 ± 0.18</td>
<td>1.75 ± 0.24</td>
</tr>
<tr>
<td>Proliferation index (%)</td>
<td>24.4 ± 0.29</td>
<td>23.7 ± 0.80</td>
<td>24.9 ± 0.57</td>
<td>42.10 ± 1.43</td>
<td>52.9 ± 1.26</td>
</tr>
</tbody>
</table>

*Significantly different from alveolar hyperplasia, bronchiolar hyperplasia, and adenoma groups (P < 0.05).
The results of this study indicate that the induction of early cell proliferation by NNK occurs with the bronchial and bronchiolar epithelial cells, but not with the alveolar epithelial cells. The observation that bronchial cell proliferation started on day 2, reached peak values on day 5, and returned to the control level by day 35 suggests that NNK-induced proliferation is not merely a response to cellular damage. Almost all of the NNK metabolic activation process is known to be completed within 24 h following carcinogen administration to mice (22). In our study, based on general histopathology, cellular injury was not observed on days 1 and 2. Similarly, Belinsky et al. (9, 11) reported that mild or infrequent cell damage caused by NNK could only be detected by electron microscopy, not by histology. Our results on bronchiolar cell hyperproliferation are consistent with the immunohistochemical localization of DNA adducts in the bronchial and bronchiolar epithelium. Our results are confirmed by the observation that a higher PCNA-labeling index (37%) was detected.

**DISCUSSION**

The proliferation index for adenomas at week 16 (Fig. 4c) was 6.90 ± 0.4% from a total of 54 adenomas in 11 mice, and this value was significantly lower (*P* < 0.05) than that observed for adenomas at week 52 (Fig. 4d). In malignant tumors, the proliferation index was 52.9 ± 1.9% for adenocarcinomas within adenoma, 54.1% for papillary growth adenocarcinoma, and 50.9% for solid growth adenocarcinoma (Fig. 4, a and b). The mean proliferation index for all malignant tumors was 52.5 ± 1.5%. The cell proliferation index was significantly higher for dysplasia within adenoma and malignant tumors than it was for adenomas (Table 2). The microfocus of dysplasia (Fig. 4e) and adenocarcinoma (Fig. 4f) in adenoma exhibited both a higher density and a higher number of PCNA immunostaining-positive cells. Fig. 4, g and h, illustrates the PCNA-positive cells in bronchiolar hyperproliferation (an epithelial region with proliferation index higher than 5% but without changes in the cell morphology) and hyperplasia lesions, respectively.

**Inhibition of Tumor Progression by Black Tea.** Black tea solution (0.6%) was administered to mice as the sole source of drinking fluid starting 16 weeks after NNK treatment. A significantly lower body weight in the tea-treated group was observed at week 20 (average body weight of 20.6 g versus 22.8 g for the controls; Fig. 5). Therefore, 0.3% black tea was used beginning week 28 until week 52. On average, the body weights of the mice in the tea treatment group were 9% lower during the period of weeks 24–52. Other than the lower body weights, the animals in the black tea-treated group remained healthy and active. At week 52, lung tumors were observed in all the mice in both the NNK-treated positive control group and the tea treatment group, but the tea treatment group had significantly lower tumor multiplicity and tumor volume (Table 3). The inhibitory action of black tea against the development of pulmonary malignant tumors is summarized in Table 4. The malignant tumor incidence and multiplicity were inhibited by 28 and 51%, respectively. Black tea treatment also reduced the proliferation index by 54% in adenomas and 24% in dysplasias within adenoma (Table 4).

**Table 3 The inhibitory effects of black tea on gross tumor incidence, multiplicity, and volume**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor incidence (%)</th>
<th>Tumor multiplicity (tumor number/mouse)</th>
<th>Tumor volume (mm³) per mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNK</td>
<td>100</td>
<td>22.5 ± 0.14</td>
<td>5.1 ± 0.17</td>
</tr>
<tr>
<td>NNK + tea</td>
<td>100</td>
<td>15.4 ± 0.17*</td>
<td>2.2 ± 0.05*</td>
</tr>
</tbody>
</table>

*Significantly different from the NNK group (*P* < 0.05), as analyzed by the Student *t* test.
bronchial and proximal bronchiolar epithelial cells in mice 6 h after treatment with NNK (14). The time-dependent relationship between DNA adduction and cell proliferation remains to be investigated. Because Clara cells are the only cell type capable of proliferation in the bronchiolar epithelia (23, 24), the observation of NNK-targeted DNA adduction and cell proliferation remains to be investigated. The apparent increase in PCNA, a commonly used marker for proliferation, is a cofactor of nucleotide excision repair (26—28). The apparent increase in PCNA labeling in NNK-induced proliferation cells are bronchiolar Clara cells. Some of the bronchiolar epithelia (23, 24), the observation of NNK-targeted DNA adduction and cell proliferation remains to be investigated. The apparent increase in PCNA labeling in NNK-induced proliferation cells are bronchiolar Clara cells. Some of these proliferative Clara cells may also be genetically altered (e.g., a mutation in the Ki-ras gene) and may be the origin of the hyperproliferative foci observed on day 35. Further development of these proliferative foci may lead eventually to tumorigenesis (25), especially in the formation of papillary growth adenocarcinoma. Alternatively, hyperproliferative cells in the foci may grow along the alveolar wall, differentiating into the bronchiolar cell type and entrapping resident alveolar type II cells (25). In either case, inhibition of the early cell hyperproliferation is expected to suppress tumor development.

In the present study, solid and papillary adenocarcinomas represented 17.8 and 19.5% of malignant tumors, respectively. The two patterns of malignant tumors exhibited similar proliferation indices. In adenocarcinoma within adenoma, 91% had the solid growth pattern, and 9% showed a mixed solid and papillary growth pattern, indicating histological heterogenicity in malignant tumors. The cellular origins of solid and papillary growth adenocarcinomas are unclear. Whether these two types of adenocarcinomas are derived independently from different cell types or whether the papillary pattern is a more advanced stage of solid tumor progression remains to be determined.

PCNA, a commonly used marker for proliferation, is a cofactor of DNA-polymerase and is required for DNA replication and DNA nucleotide excision repair (26—28). The apparent increase in PCNA immunostaining-positive cells is a general feature of tumor progression. Hyperproliferation of microfoci occurred in adenomas that are categorized as microdysplasia or adenocarcinoma. Even in adenomas with no dysplasia, the PCNA labeling index was much higher at week 52 than at week 16, suggesting that there was an increased cell proliferation with tumor progression.

Previous studies conducted by us and others have indicated that the administration of black and green teas or tea polyphenols such as EGCG inhibited NNK-induced lung adenoma formation (17, 18). The present study demonstrates that even after the lung adenomas were formed (at week 16 when there was a 100% adenoma incidence in a parallel group of mice), administration of black tea was still effective in inhibiting the progression of adenomas to adenocarcinomas (Table 4). Black tea inhibited both the malignant tumor incidence and multiplicity. These inhibitory actions of black tea may be enacted, at least in part, by inhibiting the cell proliferation in adenomas. The cell proliferation indices in the lesions with adenoma and dysplasia were decreased significantly in the black tea treatment group. This result, together with the suppression of NNK-induced early hyperproliferation by black tea polyphenols, suggests that the antiproliferative activity is important in the inhibitory action of black tea against carcinogenesis. The effective components in black tea responsible for this activity are not known. Because the black tea polyphenol preparation showed an antiproliferative effect (Table 1), theaflavins, catechins, and other unidentified constituents may all contribute to the antiproliferative and anticarcinogenic activity of black tea.

In the long-term experiments, the body weights of the animals in the tea treatment group on average were about 10% lower than those in the control group (Fig. 5). It is not known whether the lower body weights contribute to the inhibition of tumor progression in the tea treatment group. It has been demonstrated that moderate caloric restriction of 20—30% below the ad libitum intake level can lower the incidence and delay onset of tumorigenesis (29—31). In our experiment, the lower body weight was not due to lower amounts of food intake. It may be a result of decreased nutrient absorption or utilization due to the tea polyphenols or of increased metabolic rates caused by caffeine.

Black tea is one of the most commonly consumed beverages worldwide. The present studies clearly demonstrate that black tea suppresses cell hyperproliferation at early and late stages of carcinogenesis and that it inhibits the progression from adenoma to adenocarcinoma in the mouse lung. These properties may be very useful for cancer prevention. Epidemiological studies concerning the relationship between tea consumption and cancer risk have been inconclusive (17). One recent report suggested that high consumption of black tea is inversely associated with the risk of lung cancer (32), whereas another study reported that tea consumption had no effect on human lung cancer risk (33). The potential use of tea in the chemoprevention of human lung cancer requires further investigation.

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