Inhibition of Tobacco-specific Nitrosamine-induced Lung Tumorigenesis in A/J Mice by Green Tea and Its Major Polyphenol as Antioxidants

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ABSTRACT

In this study we examined the effects of green tea and its major components, (-)-epigallocatechin gallate (EGCG) and caffeine, on the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK)-induced lung tumorigenesis in A/J mice. We also studied the effects of green tea and EGCG on O6-methylguanine and 8-hydroxydeoxyguanosine (8-OH-dGuo) formation in lung tissues caused by NNK treatment. Mice were given 2% tea, 560 ppm EGCG, or 1120 ppm caffeine in drinking water for 13 weeks. During this time, NNK (11.65 mg/kg body weight) was administered by gavage three times weekly for 10 weeks from weeks 3 to 12. The bioassay was terminated 6 weeks after the last NNK treatment. Mice treated with NNK developed 22.5 lung adenomas per mouse, whereas NNK-treated mice that drank green tea or EGCG as drinking water developed only 12.2 (P < 0.01) and 16.1 (P < 0.05) tumors per mouse, respectively. Mice that drank green tea or caffeine solution showed lower body weight gains, although little difference in water and diet consumption was noted in these groups. While green tea and EGCG exerted little effect on the formation of O6-methylguanine, a critical DNA lesion in NNK lung tumorigenesis, both treatments suppressed the increase of 8-OH-dGuo levels in mouse lung DNA. The inhibition of 8-OH-dGuo formation in lung DNA by green tea and EGCG is consistent with their ability to inhibit lung tumorigenesis by NNK. Because 8-OH-dGuo is a DNA lesion induced by oxidative damage, these results suggest that the mechanism of inhibition by green tea and EGCG in NNK-induced lung tumorigenesis is due at least partly to their antioxidant properties.

INTRODUCTION

Among the carcinogens found in cigarette smoke, NNK4 appears highly specific for lung cancer induction in various laboratory animals (1, 2). NNK is also one of the most potent carcinogenic nitrosamines found in tobacco (2). These activities suggest that NNK may play a significant role in the development of lung cancer in smokers. Our studies showed that dietary compounds such as isothiocyanates and indoles in cruciferous vegetables inhibited lung tumor induction in rats and mice treated with NNK (3, 4). Furthermore, NNK-treated mice fed the cereal-based diet (NIH-07) developed significantly fewer lung tumors than those fed the semipurified diet (AIN-76A) (5). These results indicate that NNK-induced lung carcinogenesis is influenced by diet. Epidemiological studies have shown that lung cancer mortality among males in Japan is considerably lower than it is in the United States even though the prevalence of cigarette smoking during the last 40 years among Japanese males is much higher than in the United States (6). Diet could be an important factor contributing to the difference in lung cancer risk between these two countries, although other factors such as race and smoking patterns may also be involved. Considering the dietary differences between these two countries, the prevalence of green tea consumption in Japan is an intriguing one because numerous studies have shown that green tea and its polyphenol extract inhibited mutagenesis and carcinogenesis (7–11). In order to assess the possible role of green tea in the protection against lung cancer, in this study we examined the effects of green tea and its major components, EGCG and caffeine (structures in Fig. 1), on NNK-induced lung tumorigenesis in A/J mice. In addition, we studied the effects of green tea and EGCG on liver and lung O6-mGua and 8-OH-dGuo formation, both of which are DNA lesions caused by NNK treatment (12, 13).

MATERIALS AND METHODS

Chemicals. NNK was synthesized by a previously published method (14). Green tea was procured from the Tea Research Institute of The Chinese Academy of Agricultural Sciences in Hangzhou, China, and stored in a sealed plastic bag at 4°C before use. EGCG was obtained by fractionation of a crude catechine mixture, which was prepared from green tea by a method described by Matsuzaki and Hará (15), using Sephadex LH-20 (Pharmacia, Inc.) column chromatography according to a method described by Takino et al. (16). The purified EGCG was stored at −20°C and its purity was determined to be greater than 99% by HPLC analysis. Caffeine was purchased from Sigma Chemical Co. (St. Louis, MO). The 2% tea was prepared daily by adding 50 ml of boiling water to 1 g of green tea leaves followed by filtration after letting the mixture stand at room temperature for 30 min. The concentrations of EGCG and caffeine in the tea infusion were determined by the reverse-phase HPLC described below. EGCG and caffeine solutions were prepared daily with tap water. The concentrations of EGCG and caffeine, 560 and 1120 ppm, respectively, were identical to those found in the tea. O6-mGua was purchased from Chemsys Science Laboratories (Lenexa, KS). Guanine, deoxyguanosine, nuclease P1, RNase, alkaline phosphatase (type III), and proteinase K were purchased from Sigma. 8-OH-dGuo, prepared by the method of Kasai and Nishimura (17) and purified by HPLC as described by Floyd et al. (18), was kindly provided by Dr. Emerich S. Fiola.

HPLC Chromatography. Concentrations of EGCG and caffeine in tea were determined on a Varian 5000 HPLC system and a Varian 2050 UV detector (Sunnyvale, CA) using a Whatman Partisil-5 ODS-2 column (Whatman, Inc., Clifton, NJ) eluted isocratically with H2O/dimethylformamide/acetic acid/acetonitrile (81/15/3/1) at a flow rate of 1.0 ml/min. O6-mGua was analyzed using a HPLC system coupled to a Perkin Elmer LS40 fluorescence detector (Norwalk, CT) and a LC290 UV spectrophotometric detector as reported previously (12). The HPLC system consists of a Perkin Elmer binary LC pump 250 and two Whatman Partisol-10 SCX (25 x 0.45 cm) columns eluted with ammonium phosphate buffer in 10% methanol (0.1 M, pH 2.0) at a flow rate of 2.0 ml/min. Analysis of 8-OH-dGuo was performed using a HPLC system connected to a model 111B UV detector and a model LC17A/LC-4B amperometric detector from Bioanalytical Systems (West Lafayette, IN), set at 1.0 A range, +600 mV (13). The HPLC system consists of a Waters model 510 HPLC pump equipped with a Waters...
model U6K injector (Milford, MA). The column used was a 0.46-x 25-cm Altex Ultrasphere ODS column (Beckman Instruments, Inc., Berkeley, CA) protected with a C18 guard column (Alltech Associates, Inc., Deerfield, IL). The eluant was a 10% aqueous methanol containing 12.5 mM citric acid, 25 mM sodium acetate, and 10 mM acetic acid (pH 5.1) and run at a flow rate of 1 ml/min. Guanine was quantitated with the HPLC-UV detection.

Animal Bioassay. Six-week-old female A/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and kept under quarantine for 2 weeks prior to the experiment. Animals were fed AIN-76A diet (5% corn oil) and kept in plastic cages, with 5 mice/cage. They were maintained under a 12-h light/dark cycle, at 20 ± 2°C (SD) and a relative humidity of 50 ± 10%. Mice were divided into 7 groups as shown in Table 1. Tea, EGCG, and caffeine solutions were test substances consumed as drinking water for 13 weeks. After consumption of test substances in drinking water for 2 weeks, mice were given NNK by gavage (56 µmol/kg body weight or 11.65 mg/kg body weight in corn oil) 3 times weekly for 10 weeks. The daily water consumption was measured and weekly body weights were recorded. Food consumption was determined only for weeks 6 and 7 after NNK treatment began. One week after the last NNK treatment, all groups were given tap water until sacrifice. Mice were sacrificed 6 weeks after the last NNK administration. Lung adenomas were counted. Representative tissues were processed for histopathological examination of tumors. Statistical significance was determined by the 2-sample Student t test.

DNA Adduct Formation. Groups of 10 to 12 six-week-old female A/J mice were given 2% tea solution, EGCG solution, or water as drinking water for 5 weeks. The concentration of EGCG solution was identical to that used in the bioassay (560 ppm EGCG in water). Two weeks after these treatments, NNK was administered in corn oil by gavage at a dose of 112 µmol/kg body weight or 23 mg/kg body weight (pH 5.1) and run at a flow rate of 1 ml/min. Guanine was quantitated with the HPLC-UV detection.

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RESULTS

Inhibition of NNK Lung Tumorigenesis. In the present study NNK was given by gavage in 30 doses over a period of 10 weeks. Each dose was 11.65 mg/kg body weight which equals a total NNK dose of 1.7 µmol/kg body weight (350 mg/kg body weight). This dose regimen induced a 100% tumor incidence with an average of 22.5 lung adenomas/mouse at week 16 after NNK treatment began. Table 1 shows the inhibitory effects of green tea, EGCG, and caffeine on the formation of lung adenomas in NNK-treated mice. While the tumor incidences were not affected by green tea and EGCG, mice that drank tea or EGCG solution developed an average of only 12.2 or 16.1 tumors/mouse. These tumor multiplicities correspond to a 45 and 30% reduction of lung tumor formation in these groups as compared with the NNK-treated group that drank water. Interestingly, the caffeine group also showed a marginal but significant inhibition. Groups that drank tea, EGCG, or caffeine without NNK treatment showed the tumor incidence and multiplicity comparable to the background levels commonly seen in the control groups of our previous studies (3).

Fig. 2 shows the body weight curves of different groups during bioassay. Independent of NNK treatment, groups that drank tea or caffeine solution showed consistently lower weight gains as compared with groups that drank water or EGCG solution. Since comparable body weight gains were observed in the tea and caffeine groups, it appears that caffeine is responsible for the decrease in weight gain in the tea group. In contrast to the tea and caffeine groups, mice that drank EGCG solution had normal growth. The weekly water consumption, 0.6–0.7 ml/g body weight, was similar among most of the groups, with only the exception of the tea group which consumed an average of 0.82 ml/g body weight. No difference in diet consumption between groups was found.

Table 1 Effect of tea, EGCG, and caffeine on NNK-induced lung adenomas in A/J mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>% of mice with tumors</th>
<th>No. of animals</th>
<th>Tumors/mouse ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNK</td>
<td>100</td>
<td>30</td>
<td>22.5 ± 4.7</td>
</tr>
<tr>
<td>Tea + NNK</td>
<td>100</td>
<td>25</td>
<td>12.2 ± 4.3*</td>
</tr>
<tr>
<td>EGCG + NNK</td>
<td>100</td>
<td>25</td>
<td>16.1 ± 5.3*</td>
</tr>
<tr>
<td>Caffeine + NNK</td>
<td>100</td>
<td>15</td>
<td>19.2 ± 4.8*</td>
</tr>
<tr>
<td>Tea</td>
<td>7</td>
<td>15</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>EGCG</td>
<td>20</td>
<td>15</td>
<td>0.3 ± 0.6</td>
</tr>
<tr>
<td>Caffeine</td>
<td>20</td>
<td>15</td>
<td>0.3 ± 0.6</td>
</tr>
</tbody>
</table>

* Statistically different from NNK group, P < 0.001.
* Statistically different from NNK group, P < 0.05.
EFFECTS OF GREEN TEA AND EGCG ON LUNG TUMORIGENESIS AS ANTIOXIDANTS

DISCUSSION

This study showed that green tea has a protective effect against NNK-induced lung tumorogenesis in A/J mice and the polyphenol EGCG in green tea appears to be a major active component for this activity. Green tea is composed of at least 10 to 20% polyphenols (23). These compounds are powerful antioxidants, capable of scavenging H\textsubscript{2}O\textsubscript{2} and superoxide anion, thus preventing H\textsubscript{2}O\textsubscript{2} and oxygen free radical-induced cytotoxicity and mutagenicity (24, 25). Green tea polyphenol fractions have been shown to be protective in mice against skin tumor induction by poly cyclic aromatic hydrocarbons (7). They also inhibited aflatoxin B<sub>1</sub> hepatocarcinogenesis (26). The administration of several types of Chinese tea as drinking water resulted in a significant reduction in esophageal tumors induced by N-nitrosobenzylmethylamine (8). Total green tea extract given p.o. also protected mice from UV-induced skin tumorogenesis (27). Because either tea or total tea polyphenol fraction was used in these studies, it is not possible to identify which are the active compounds in green tea responsible for the inhibitory effect. EGCG, the main polyphenol in green tea, is an antimutagen (9). Topical application of EGCG-inhibited telocidcine promoted 7,12-dimethylbenz(a)anthracene-induced skin tumors in mice (28). More recently, the antipromoting activity of EGCG was demonstrated in mouse duodenum carcinogenesis induced by N-ethyl-N-nitro-N-nitrosoguanidine (10). Our results showed that EGCG, given in a concentration identical to that found in tea infusion, inhibited NNK-induced lung tumorogenesis in mice. Furthermore, EGCG, unlike caffeine, exerted no adverse effect on growth. Therefore, it may be considered as a potential chemopreventive agent. It is estimated that an average smoker is exposed to roughly 2 mg of NNK annually (29). In our bioassay, mice consumed an average of 100 mg of EGCG and at the same time were exposed to 7 mg of NNK (EGCG:NNK, 14:3). Thus, one may hypothesize that if the inhibitory activities of EGCG are important in humans, a cup of tea per day could represent a protective effect against NNK-induced lung tumorogenesis in A/J mice.

Effects of O\textsuperscript{6}-mGua and 8-OH-dGuo Formation. Multiple NNK doses resulted in an accumulation of O\textsuperscript{6}-mGua in both lung and liver DNA. Table 2 shows that, 2 h after the last NNK treatment at a dose of 23 mg/kg body weight, levels of O\textsuperscript{6}-mGua in mouse lung and liver were 55.3 and 107 \textmu mol/mol guanine, respectively. Despite the fact that O\textsuperscript{6}-mGua is a critical lesion in NNK tumorogenesis in A/J mice (20–22), tea or EGCG solution given as drinking water before and during NNK administration exhibited little effect on the formation of this DNA lesion in either lung or liver. In a separate study, O\textsuperscript{6}-mGua in lung DNA was measured at 4 and 24 h after NNK treatment. Table 3 shows that levels of O\textsuperscript{6}-mGua were not significantly affected at both time points by green tea or EGCG treatment. In fact, a small increase of O\textsuperscript{6}-mGua in the tea group was seen as compared with the NNK control group 4 h after NNK treatment. Little difference in O\textsuperscript{6}-mGua levels between 4 and 24 h in both tea and EGCG groups suggests that the O\textsuperscript{6}-mGua methyltransferase activity was not altered by these treatments. Table 4 shows that multiple doses of NNK caused a significant increase in the 8-OH-dGuo levels in lung DNA from 1.7 ± 1.2 to 3.2 ± 1.7 adducts/10\textsuperscript{5} deoxyguanosine, an approximately 2-fold elevation from the basal levels. Only a slight increase from 3.9 ± 0.6 to 4.7 ± 1.1 adducts/10\textsuperscript{5} deoxyguanosine was seen in liver DNA; however, this increase was not statistically significant. Green tea and EGCG suppressed the elevated 8-OH-dGuo levels in lungs of NNK-treated mice. The decrease in 8-OH-dGuo levels in lungs of NNK-treated mice that drank green tea or EGCG is consistent with their inhibitory activity against lung tumor formation.

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Table 2 Effects of green tea and EGCG on O\textsuperscript{6}-mGua formation in lung and liver DNA of A/J mice 2 h after treatment with NNK\textsuperscript{*}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Lung (\textmu mol/mol guanine)</th>
<th>Liver (\textmu mol/mol guanine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNK</td>
<td>10</td>
<td>55.3 ± 11.0\textsuperscript{*}</td>
<td>107.7 ± 29.8</td>
</tr>
<tr>
<td>NNK + Tea</td>
<td>12</td>
<td>57.6 ± 16.7</td>
<td>110.1 ± 38.2</td>
</tr>
<tr>
<td>NNK + EGCG</td>
<td>12</td>
<td>57.5 ± 12.3</td>
<td>94.2 ± 36.7</td>
</tr>
</tbody>
</table>

\textsuperscript{*} Statistically different from NNK group, P < 0.01.

Table 3 Effects of green tea and EGCG on O\textsuperscript{6}-mGua levels in lung DNA of A/J mice 4 and 24 h after treatment with NNK\textsuperscript{*}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>O\textsuperscript{6}-mGua (\textmu mol/mol guanine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 h</td>
<td>24 h</td>
</tr>
<tr>
<td>NNK</td>
<td>168 ± 2.7\textsuperscript{*}</td>
</tr>
<tr>
<td>NNK + tea</td>
<td>233 ± 2.8</td>
</tr>
<tr>
<td>NNK + EGCG</td>
<td>196 ± 1.0</td>
</tr>
</tbody>
</table>

\textsuperscript{*} Statistically different from control group, P < 0.01.

Table 4 Effects of green tea and EGCG on the 8-OH-dGuo levels in lung and liver DNA of A/J mice 2 h after treatment with NNK\textsuperscript{*}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>8-OH-dGuo (adducts/10\textsuperscript{5} deoxyguanosine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice</td>
<td>Lung</td>
</tr>
<tr>
<td>Control</td>
<td>11</td>
</tr>
<tr>
<td>NNK</td>
<td>10</td>
</tr>
<tr>
<td>NNK + tea</td>
<td>11</td>
</tr>
<tr>
<td>NNK + EGCG</td>
<td>12</td>
</tr>
<tr>
<td>EGCG</td>
<td>10</td>
</tr>
<tr>
<td>Tea</td>
<td>11</td>
</tr>
</tbody>
</table>

\textsuperscript{*} Statistically different from control group, P < 0.01.
of green tea per day consumed by humans would appear to contain a sufficient amount of EGCG to alleviate the potential carcinogenic action of NNK.

In the tea preparation used in the present study, caffeine constitutes about 5.6% of tea leaves by dry weight. It is a major component in tea and has been shown to inhibit chemical carcinogenesis in other studies (30–32). The exact mechanisms by which caffeine inhibits the induction of tumors are not clear. Recently, Lagopoulos et al. (33) found that the reduced body weight gains in caffeine-treated groups correlated with decreased hepatocarcinogenesis by diethylnitrosamine. Vander-Ploeg et al. (34) suggested that the inhibition of 7,12-dimethylbenz[a]anthracene-induced mammary gland tumorigenesis in rats by caffeine was due to the ability of caffeine to suppress its metabolism. In the present study, although diet consumption appeared to be unaffected by caffeine treatment, the body weight gains in the caffeine group were considerably lower than those in the EGCG and NNK control groups. Therefore, the slight but significant reduction in lung tumor multiplicity by caffeine treatment could be related to its negative effect on body weight. Regardless of the mechanism, the potential protective effect of caffeine should not be ignored because of its widespread consumption by humans.

Several lines of evidence indicated that O6-mGua is a critical lesion in NNK lung tumorigenesis (20–22). Previously we showed that a decrease in lung O6-mGua formation correlated with a decrease of lung tumor formation in NNK-treated A/J mice pretreated with arylalkyl isothiocyanates or indole-3-carbinol (4, 35). In the present study, however, neither green tea nor EGCG treatment inhibited NNK-induced O6-methylguanine formation or stimulated its repair, although both treatments inhibited the lung tumor induction by NNK. These results suggested that additional mechanisms other than DNA methylation or repair are likely to be involved in lung tumorigenesis by NNK. NNK treatment also resulted in pyridyloxobutane adduct of deoxyguanosine: sensitive detection and mechanism of formation. Free Radical Res. Commun., 1: 163–172, 1986.


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