(-)-Epigallocatechin gallate can prevent cisplatin-induced lung tumorigenesis in A/J mice

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Introduction

Green tea contains a variety of polyphenols known as catechins. (-)-Epigallocatechin gallate (EGCG) is a major component of polyphenols in green tea. Polyphenols in green tea have anti-oxidative (1,2), anti-mutagenic (3,4), anti-carcinogenic (5) and antitumor (6) activities in experimental animals. Epidemiological studies have shown a lower risk of cancer among people who consume a large amount of green tea (7,8).

The global incidence of lung cancer is increasing at a rate of 0.5% per year and, as a consequence, lung cancer is a leading cause of cancer mortality in most countries (9). The introduction of combined modality therapy (chemotherapy and cisplatin-based chemotherapy) has produced disease-free long-term survival in lung cancer (10,11). Second primary cancers have increased in small-cell lung cancer (12). The risk of secondary lung cancer in patients with non-small cell lung cancer is estimated to be 1–2% per patient per year (13). For patients with small-cell lung cancer, the risk is increased to >2–10% per patient per year 10 years after the initial treatment (13). Continued smoking, chemotherapy and chest radiation affect the incidence of secondary lung cancer. These factors will be critical in the prevention of secondary lung cancer. Since free platinum intercalates or intracalates in DNA, cisplatin can be cytotoxic towards lung cancer cells, but it can also enhance carcinogenicity in experimental animals (14). EGCG can prevent 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumorigenesis; it does this by inhibiting the formation of O6-methylguanine adducts caused by NNK (15) and protecting from oxidative damage through inhibition of 8-hydroxydeoxyguanosine (16), and also has a direct anti-tumor effect (6). Accordingly, we presumed that EGCG might protect from cisplatin-induced DNA damage and/or inhibit tumor growth directly, and tested whether EGCG could prevent cisplatin-induced tumorigenesis in a mouse model.

Materials and methods

Animals and chemicals

A total of 132 female A/J mice (Japan SLC, Shizuoka, Japan), 4 weeks old and weighing approximately 17 g, were used. The animals were housed, five per plastic cage, and were given free access to tap water and standard laboratory food (MF; Oriental Yeast, Tokyo, Japan). They were kept in an air-conditioned room with 55 ± 10% humidity under a daily cycle of alternating 12 h periods of light and darkness in the Animal Center for Medical Research, Okayama University Medical School, Oriental Menhol Industry (Okayama, Japan) and nippon Kayaku (Tokyo, Japan) kindly provided EGCG (>75% purity) and cisplatin, respectively. NNK and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyketrazolium bromide (MTT) were purchased from Toronto Research Chemicals (Ontario, Canada) and Sigma (St Louis, MO, USA), respectively. EGCG was provided in drinking water when indicated in the experiment.

Experimental design

Experiment 1. Fifty mice were divided into two groups, for treatment with NNK (n = 25) or with NNK and EGCG (n = 25). From 1 week after starting EGCG treatment (1 mg/ml in tap water), mice were treated with NNK (1 mg/kg body wt, i.p.) twice a week for 28 weeks. All mice were killed at week 30 and the number of lung tumors per mouse was determined.

Experiment 2. Eighty-two mice were divided into four groups as controls (group 1; no treatment; n = 20) or for treatment with EGCG (group 2; n = 20), cisplatin (group 3; n = 22) or cisplatin and EGCG (group 4; n = 20). After 2 weeks of EGCG treatment, mice were treated with cisplatin (1.62 mg/kg body wt, i.p.) once a week for 18 weeks. At week 30 (18 weeks after the last cisplatin treatment), all mice were killed and the number of lung tumors per mouse was determined.

Cell culture and cytotoxicity test

The two non-small cell lung cancer cell lines A549 (JCRB0076) and LK-2 (JCRB0829) were provided by the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan). The small-cell lung cancer cell line SBC-3 (JCRB0818) was established in our laboratory (17). The growth medium was RPMI-1640 supplemented with 10% fetal bovine serum, penicillin-G (100 U/ml) and streptomycin (100 μg/ml). Cytotoxicity was evaluated by an MTT assay, which was modified from the original method reported by Mosmann (18), as
cisplatin and EGCG. Therefore, EGCG significantly prevented weight loss in mice treated with cisplatin after week 16. One of 20 mice in the cisplatin group died (at week 12). The primary causes of death were not investigated.

Table II shows tumor incidence and multiplicity in each group. Tumor incidence was 90.9% (20/22) in the NNK group but only 29.2% (7/24) in the group treated with NNK and EGCG (Table I). In the mice treated with cisplatin, 4.6 ± 2.4 (mean ± SD) lung tumors were found per mouse, whereas in NNK-treated mice that drank EGCG in drinking water, there were only 0.6 ± 1.1 tumors per mouse (P < 0.001). After week 12, the mice in the NNK group weighed significantly less than those treated with NNK and EGCG group (Figure 1). Three of the 25 mice in the NNK group died (one each at weeks 16, 24 and 24). One of 25 mice in the NNK plus EGCG group died (at week 12). The primary causes of death were not investigated.

Table II shows tumor incidence and multiplicity in each group. Tumor incidence was 100% (19/19) and 94.4% (17/18) in groups 3 (cisplatin treatment) and in 4 (cisplatin plus EGCG treatment), respectively. Thus, EGCG treatment did not reduce tumor incidence. However, in mice treated with cisplatin, 5.1 ± 2.1 (mean ± SD) lung tumors developed per mouse, compared with only 2.8 ± 2.5 per mouse in those treated with cisplatin and EGCG. Therefore, EGCG significantly reduced the number of cisplatin-induced lung tumors in A/J mice (P < 0.01). There were no differences in tumor incidence and multiplicity between groups 2 (EGCG treatment) and 1 (control). EGCG alone did not reduce the incidence or multiplicity of spontaneous lung tumors in A/J mice.

Pulmonary tumors were well demarcated and composed of cord-like structures with scattering tubules (hematoxylin and eosin stain ×300). (B) A higher magnification of panel A. Tumor cells have dysplastic nuclei of various shapes and sizes. Mitotic figures are indicated with white arrowheads (hematoxylin and eosin stain ×600).

**Table II. The inhibitory effects of cisplatin on gross tumor incidence and multiplicity in A/J mice**

<table>
<thead>
<tr>
<th></th>
<th>Cisplatin</th>
<th>Cisplatin and EGCG</th>
<th>EGCG</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of mice</td>
<td>19</td>
<td>18</td>
<td>20</td>
<td>19</td>
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<tr>
<td>No. of tumor-bearing mice</td>
<td>19</td>
<td>17</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Tumor incidence(%)</td>
<td>100</td>
<td>94.4</td>
<td>30</td>
<td>26.3</td>
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<tr>
<td>Tumor multiplicity</td>
<td>5.1 ± 2.1</td>
<td>2.8 ± 2.3</td>
<td>0.4 ± 0.8</td>
<td>0.4 ± 0.8</td>
</tr>
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</table>

*aStatistical significance (determined by Student’s t-test): cisplatin versus cisplatin and EGCG, P < 0.01; for cisplatin versus EGCG, cisplatin versus control, cisplatin and EGCG versus EGCG, and cisplatin and EGCG versus control, P < 0.001; for EGCG versus control, not significant.

**Results**

Tumor incidence was 90.9% (20/22) in the NNK group but only 29.2% (7/24) in the group treated with NNK and EGCG (Table I). In the mice treated with cisplatin, 4.6 ± 2.4 (mean ± SD) lung tumors were found per mouse, whereas in NNK-treated mice that drank EGCG in drinking water, there were only 0.6 ± 1.1 tumors per mouse (P < 0.001). After week 12, the mice in the NNK group weighed significantly less than those treated with NNK and EGCG group (Figure 1). Three of the 25 mice in the NNK group died (one each at weeks 16, 24 and 24). One of 25 mice in the NNK plus EGCG group died (at week 12). The primary causes of death were not investigated.

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Pulmonary tumors were well demarcated and neoplastic cells proliferated with cord-like and/or papillotubular structures (Figure 2A). A higher magnification revealed that tumor cells had polygonal cytoplasm with nuclei of various shapes and sizes. Mitotic figures were easily observed (Figure 2B). These tumors were classified as an adenocarcinoma according to the description of Yang et al. (21). Some smaller tumors were composed of cells with rather lower anaplasia, which corresponded to adenoma.

Treatment with EGCG alone did not lead to significant weight gain compared with controls. Mice in group 3 (cisplatin treatment) began to lose significant weight (compared with the controls) 2 weeks after starting cisplatin treatment (P < 0.05 at week 2; P < 0.01 at weeks 4, 6 and 8), but mice in group 4 (treated with cisplatin and EGCG) did not lose weight during the 10-week period after administration of cisplatin. As shown in Figure 3, EGCG significantly prevented weight loss in mice treated with cisplatin after week 16. One of 20 mice in the

**Table I. The inhibitory effects of EGCG on gross tumor incidence and multiplicity in A/J mice**

<table>
<thead>
<tr>
<th></th>
<th>NNK</th>
<th>NNK and EGCG</th>
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<tbody>
<tr>
<td>Total no. of mice</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>No. of tumor-bearing mice</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>Tumor incidence(%)</td>
<td>90.9</td>
<td>29.2</td>
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<tr>
<td>Tumor multiplicity</td>
<td>4.6±2.4</td>
<td>0.6±1.1</td>
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</table>

*aStatistical significance (determined by χ² test) for NNK compared with NNK plus EGCG, P < 0.001.

*bNumber of tumors per mouse. Statistical significance (determined by Student’s t-test) for NNK compared with NNK plus EGCG was P < 0.001.
EGCG can prevent cisplatin-induced lung tumors

Discussion

It has been reported that pulmonary adenomas occur in A/J mice 18 weeks after cisplatin treatment (14). Using this system, we demonstrated that tumor multiplicity was significantly reduced by administering EGCG to cisplatin-treated mice ($P < 0.01$). EGCG induces G2-M arrest and inhibits growth of tumor cell lines (22). It also inhibits production of urokinase, which can decrease tumor size or even produce complete remission of cancer in mice (23). These observations suggest that direct effects of EGCG may contribute to the delay of tumor formation and/or progression. Several experimental studies have demonstrated that green tea polyphenols and/or EGCG can inhibit the incidence of chemically induced tumors in the duodenum (24), colon (25), skin (26,27) and lung (16). We have confirmed that EGCG can inhibit NNK-induced lung tumorigenesis in A/J mice. NNK in cigarette smoke is a potent carcinogen (28), which is related to both secondary and primary lung cancer (29). Cisplatin used in the chemotherapy of lung cancer is also a potent carcinogen (14), which is probably related to secondary lung cancer. We confirmed that EGCG has a chemopreventive effect on NNK-induced tumors and demonstrated a partial effect of EGCG on cisplatin-induced tumors. EGCG thus appears to be a good candidate for chemoprevention of secondary lung cancer.

EGCG decreased both tumor incidence and multiplicity in NNK-induced tumors, whereas it only affected multiplicity in cisplatin-induced tumors. It may act by metabolic inactivation of NNK as well as by direct inhibition of tumor growth. An activated K-ras gene with a specific transition (GGT to GAT) in codon 12, which is generated through the formation of O6-methylguanine adducts by NNK, is frequently detected in NNK-induced lung tumors (30). EGCG can inhibit the formation of O6-methylguanine adducts (15) and thus prevent lung tumors related to an activated ras gene. Although the mechanism of cisplatin-induced tumorigenesis in A/J mice is not well established, EGCG may not inactivate cisplatin directly like NNK in the first stage in carcinogenesis, because EGCG did not reduce the antitumor activity of cisplatin in vitro. EGCG may inhibit tumor growth directly rather than protect from cisplatin-induced DNA damage.

EGCG prevented only additional tumors induced by cisplatin; it did not prevent spontaneous lung tumors. Belinsky et al. have reported that cisplatin reduces NNK-induced cancer multiplicity (31). In their experiment, cisplatin treatment was initiated 42 weeks after NNK treatment. At this time point, adenocarcinoma of the lung induced by NNK already exists and can be treated by cisplatin. Furthermore, mice were killed after 8 weeks of cisplatin treatment. This follow-up period is too short to detect carcinogenicity of cisplatin. In their system, the effects of cisplatin as an anticancer drug could overcome its effects as a carcinogen.

In the first experiment, NNK (1 mg/kg body wt) was injected intraperitoneally into female A/J mice twice a week for 28 weeks. Low-dose multiple injections of NNK for 28 weeks induced a high tumor incidence (90.9%) with a mean of 4.6 tumors in the lungs. Although the incidence and multiplicity are low when compared with the previous report (16), low-dose, long-term exposure of NNK is closer to the clinical condition, since NNK is a carcinogen in tobacco smoke. EGCG effectively inactivated low-dose NNK and thus markedly reduced both incidence and multiplicity of lung tumors to the background level in A/J mice. Although cachexia-related...
cytokines, such as tumor necrosis factor and interleukins 1 and 6 (32), were not checked after week 12, significant weight loss in the NNK treatment group during this phase may have been related to NNK-induced tumors themselves rather than the direct NNK toxicity.

EGCG prevented cisplatin-induced weight loss during early and late phases. In the late phase (after week 16), EGCG delayed tumor progression and, as a consequence, might have prevented tumor-related weight loss, as observed in the NNK treatment group. In the early phase of cisplatin administration, EGCG reduced weight loss due to cisplatin's toxicity. EGCG may protect normal cells in the mice from adverse effects of cisplatin, because the mice did not have tumors in this phase. It is not clear why mice treated with cisplatin and EGCG did not lose weight.

Green tea, which contains EGCG, is a natural product and has no harmful effects. However, a large amount of green tea may have minor adverse effects such as epigastric discomfort and sleeplessness caused by caffeine. For clinical application, it is necessary to reduce the adverse effects and to amplify the chemopreventive effect (33). Iwata (34) has reported that purified EGCG is too expensive to be mass produced. For clinical use, crude green tea extract can be produced easily and cheaply. Decaffeinated green tea extract will be useful for reducing adverse effects in clinical application (34).

In conclusion, EGCG partially inhibited cisplatin-induced lung tumorigenesis and weight loss in A/J mice. Our observations suggest that EGCG may prevent the secondary malignancy and weight loss caused by cisplatin and/or tumors themselves.

Acknowledgements
We thank Drs Tadashi Tsuchida, Hiromichi Yamane, Naoyuki Nogami and Akio Hiraki for their excellent technical assistance and helpful suggestions.

References


*Received June 7, 1999; revised December 16, 1999; accepted December 17, 1999*