Suppressive Effect of (–)-Epigallocatechin Gallate on Aflatoxin B₁-induced Chromosome Aberrations in Rat Bone Marrow Cells

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The suppressive effect of (–)-epigallocatechin gallate (EGCG), the major polyphenolic constituent present in green tea, on aflatoxin B₁ (AFB₁)-induced chromosome aberrations (CA) in rat bone marrow cells was studied. The administration of EGCG 24 hr before the AFB₁ injection significantly suppressed AFB₁-induced CA. The suppression was observed 18 hr, 24 hr and 48 hr after the AFB₁ treatment but no suppressive effect was observed at the early period (6 hr and 12 hr) after the AFB₁ treatment. Furthermore, the suppression was observed in all doses of AFB₁ (1, 5, 10 and 20 mg/kg) investigated. Rats given EGCG 2 hr before the AFB₁ injection displayed no suppressive effect. The suppressive effect of EGCG paralleled the dose of EGCG when given in a dose range of 10–60 mg/kg body weight. The administration of (–)-epicatechin gallate 24 hr before the AFB₁ injection significantly suppressed AFB₁-induced CA as well as EGCG. On the other hand, in rats given green tea polyphenols (GTP) 2 hr before the AFB₁ injection, (–)-epigallocatechin and gallic acid significantly suppressed AFB₁-induced CA. The pretreatment with EGCG or gallic acid did not induce the drug-metabolizing enzymes in rat liver, such as cytochrome P450 and glutathione S-transferase. Rats given 2% green tea infusion as the sole source of drinking water for four days before sacrifice displayed significantly suppressed AFB₁-induced CA. However, rats given various kinds of canned tea for four days showed no suppressive effect. The amount of GTP in canned tea determined by high performance liquid chromatography (HPLC) analysis was much less than that in 2% green tea infusion.

Key words —— (–)-epigallocatechin gallate, aflatoxin B₁, chromosome aberration, rat bone marrow cell, green tea polyphenol, canned tea

INTRODUCTION

One of the most obvious ways to prevent the development of human cancer is to remove the causative agents from our environment, including our daily food. However, there are a considerable number of suspect carcinogens in the environment that have been identified by virtue of mutagenicity tests.1-4 It seems to be exceedingly difficult to remove them from our environment. Therefore, cancer chemoprevention assumes considerable importance. Cancer chemoprevention means that the occurrence of cancer is prevented by administration of one or several chemical compounds. If chemopreventive agents were contained in our daily food or drink, it would be fortunate to human beings.

On the other hand, epidemiological studies have shown a protective effect of green tea consumption against certain types of cancers.5-9 It has been reported by many researchers that the hot water extract from green tea (GTE) and green tea polyphenols (GTP) have anti-mutagenic activity10-16 or anti-tumor promotion activity.17-19 We also previously reported that the administration of GTE or GTP mixture before the aflatoxin B₁ (AFB₁) injection suppressed AFB₁-induced chromosome aberrations (CA).20 Furthermore, there are also many reports that demonstrate the anti-cancer property of GTE or GTP with in vivo animal tests.21-31

In this study, we investigated the suppressive effect of (–)-epigallocatechin gallate (EGCG), the major polyphenolic constituent present in green tea, on AFB₁-induced CA in rat bone marrow cells. The suppressive effect of other GTP, such as (–)-epicatechin gallate (ECC), (–)-epigallocatechin (EGC), (–)-epicatechin (EC), (+)-catechin (C) and gallic acid (GA), was also examined (for chemical structures, see Fig. 1). Furthermore, the suppressive
effect of 2% green tea infusion and some kinds of canned tea on AFB1-induced CA was investigated and the concentration of GTP in them was determined by high performance liquid chromatography (HPLC) analysis.

MATERIALS AND METHODS

Chemicals —— EGCG, ECG, EGC, EC and C were purchased from Kurita Kogyo Co., (Tokyo, Japan). GA and colchicine were obtained from Wako Pure Chemicals Co., (Tokyo, Japan); AFB1 was from Makor Chemicals, Ltd., (Tokyo, Japan); and DMSO (spectrophotometric grade) was from E. Merck A.G., (Darmstadt, F.R.G.).

Preparation of 2% Green Tea Infusion —— 20 g green tea leave (Karigane Sencha, Ito-En Co., Tokyo, Japan) was added to 1 l of boiling water and was steeped for 10 min. The infusion was cooled to room temperature and then filtered to obtain 2% green tea infusion (2 g green tea leave/100 ml of water).

Canned Tea —— Five kinds of canned tea (A, B, C, D and E) were purchased from a supermarket in Kobe city. A, B and C are canned green tea produced respectively by the different companies. D is canned mixed tea that brewed various kinds of tea leaves containing green tea leaves. E is canned oolong tea.

Animal Experiment —— Male rats of the Wistar strain (Charles River Japan, Inc., Kanagawa, Japan), aged 28–35 days and weighing 80–110 g, were used. Each experimental group consisted of 6 rats. They were kept in an air-conditioned room and fed MF (Oriental Ferment Co., Tokyo, Japan) and water ad libitum. AFB1 were dissolved in DMSO and injected i.p. In the first experiment, individual GTP was dissolved in water and administered by gastric instillation to lightly ether-anesthetized rats at various times before the AFB1 injection. Colchicine (0.3 mg/rat) was injected i.p. 1 hr before sacrifice. Chromosome specimens were prepared from the femoral bone marrow by the conventional method at various times after the AFB1 injection, stained in 2% Giemsa solution (pH 6.8) for 15 min, and then analyzed microscopically. In experiment 2, rats were administered 2% green tea infusion or canned tea as the sole source of drinking water for four days before sacrifice. The other protocol is the same as that in experiment 1.

Chromosome Analysis —— Metaphase cells with one or more CA were scored from 50 well-spread metaphases per rat (therefore 300 metaphases per each experimental group). Gaps were defined as achromatic lesions in one or both chromatids not exceeding the width of a chromatid, and breaks as a discontinuity greater than the width of a chromatid, irrespective of whether or not the distal fragment was dislocated. Cells with multiple CA were defined as cells in which the number of CA was too great to count (numerous, above 10). Cells were classified according to the most severe damage out of CA which had occurred in a cell and were placed in only 1 of 4 categories: cells with gaps only, cells with breaks, cells with exchanges, and cells with multiple CA. In the tabulated data, the column headed “percentage of aberrant cells” gives the percentage of damaged cells in the total population of cells analyzed. Damaged cells include the cells with breaks, exchanges and multiple CA, but not the cells with gaps. The severity of damage within a cell is also given as the number of aberrations per cell; cells with multiple CA were counted as 10 aberrations. The suppression rate was calculated from the frequency of aberrant cells.

Measurement of Cytochrome P450 Content and Glutathione S-Transferase (GST) Activity —— The hepatic microsome and cytosol fractions of rats given EGCG or GA were prepared as previously described. The cytochrome P450 content of hepatic microsomes was determined by the method of Omura and Sato on the basis of the CO-difference
The chromatography was monitored at 280 nm. The 
were centrifuged at 2600 rpm for 5 min and 10 
2% green tea infusion and some kinds of canned tea 
30 to 50 min (15% acetonitrile), 50 to 60 min (50% 
file was as follows: 0 to 30 min (9% acetonitrile), 
ated with a stepwise gradient mode. The elution pro-
buffer (pH 2.6) and acetonitrile] which were oper-
ODS-80Tm (TOSOH) (4.6 mm × 15 cm) was used 
45°C with 1.0 ml/min flow rate. The mobile phase 
Determination of GTP by HPLC Analysis ————
The concentration of individual GTP in 2% green 
tea infusion and various kinds of canned tea were 
determined by HPLC analysis using a Shimadzu LC- 
6A system with SPD-6A UV detector. A column 
ODS-80Tm (TOSOH) (4.6 mm × 15 cm) was used at 
45°C with 1.0 ml/min flow rate. The mobile phase 
contained two solvents [10 mM sodium phosphate 
buffer (pH 2.6) and acetonitrile] which were operated 
with a stepwise gradient mode. The elution pro-
file was as follows: 0 to 30 min (9% acetonitrile), 
30 to 50 min (15% acetonitrile), 50 to 60 min (50% 
acetonitrile), and 60 to 80 min (9% acetonitrile). The 
2% green tea infusion and some kinds of canned tea 
were centrifuged at 2600 rpm for 5 min and 10 µl of 
the supernatant were injected onto the column. The 
chromatography was monitored at 280 nm. The 
retention times of EGC, C, EC, EGCG and ECG were 8.9, 10.9, 20.5, 23.8 and 42.5 min, respectively.

Table 1. Variation in Course of Time of AFB1-Induced CA in the Bone Marrow Cells of Rats Receiving only AFB1 Injection or Both EGCG Pretreatment and AFB1 Injection

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Treatment</th>
<th>Percentage of cells with Gap Break Ex. Multi.</th>
<th>No. of aberration per cell</th>
<th>Percentage of aberrant cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Non-AFB1</td>
<td>1.8 ± 0.5</td>
<td>0.8 ± 1.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>6</td>
<td>AFB1 only</td>
<td>8.5 ± 3.4</td>
<td>16.1 ± 5.8</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>12</td>
<td>AFB1 only</td>
<td>10.0 ± 2.6</td>
<td>22.0 ± 4.0</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>18</td>
<td>AFB1 only</td>
<td>13.6 ± 2.3</td>
<td>28.2 ± 4.3</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>24</td>
<td>AFB1 only</td>
<td>9.2 ± 2.0</td>
<td>21.7 ± 2.1</td>
<td>0.3 ± 0.5</td>
</tr>
<tr>
<td>48</td>
<td>AFB1 only</td>
<td>10.6 ± 1.7</td>
<td>15.4 ± 4.9</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>72</td>
<td>AFB1 only</td>
<td>5.1 ± 2.3</td>
<td>7.5 ± 3.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>EGCG + AFB1</td>
<td>3.2 ± 1.8</td>
<td>2.8 ± 0.8</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>EGCG + AFB1</td>
<td>2.5 ± 1.0</td>
<td>1.5 ± 1.5</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

a) Chromosome specimens were prepared at various times after 10 mg AFB1/kg body weight was injected. A dose of 60 mg EGCG/kg body weight was given 24 hr before the AFB1 injection. Values are means ± S.D. for 6 rats. b) Ex., exchange; Multi., multiple CA: cells having more than 10 aberrations. c) Cells with gaps are not included in the percentage of aberrant cells. Figures in the parenthesis indicate the suppression rate. Significantly different from the rat group given only AFB1: **p < 0.01, *p < 0.05.

RESULTS

AFB1-induced CA consisted mainly of gaps and breaks. Cells with multiple CA or exchanges were observed infrequently. On the other hand, the rats which had received DMSO without AFB1 showed only a few gaps and breaks in their bone marrow cells. Cells with exchange or multiple CA were not observed. The frequency of aberrant cells in the bone marrow of rat injected AFB1 became higher with the lapse of time as did the number of aberrations per cell. They were at their maximum levels 18 hr after the AFB1 injection. After that, they decreased with the lapse of time (Table 1, Fig. 2). The frequency of aberrant cells induced by AFB1, increased in proportion to the dose of AFB1 (Fig. 3).

Rats given EGCG 24 hr before the AFB1 injection displayed a considerably suppressed frequency of AFB1-induced CA in their bone marrow cells. The suppression was observed 18 hr, 24 hr and 48 hr after the AFB1 injection but no suppressive effect was observed at the early period (6 hr and 12 hr) after the AFB1 injection. After that, they decreased with the lapse of time (Table 1, Fig. 2). The frequency of aberrant cells induced by AFB1, increased in proportion to the dose of AFB1 (Fig. 3).

Rats given EGCG 2 hr before the AFB1 injection showed no suppressive effect and the maximum suppression was observed in rats given EGCG 24 hr.
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before the AFB1 injection (Fig. 4). The suppressive effect of EGCG paralleled the dose of EGCG when given in a dose range of 5–60 mg/kg body weight; higher doses (180 mg/kg) produced no additional suppression (Fig. 5). Rats given only EGCG (180 mg/kg) without carcinogen displayed no induction of CA in their bone marrow cells (data not shown).

The suppressive effect of individual GTP on AFB1-induced CA is shown in Table 2. In rats given GTP 24 hr before the AFB1 injection, EGCG and

Fig. 2. Variation of the Frequency of Aberrant Cells in Bone Marrow Cells of Rats (●) Receiving only the AFB1 Injection or Rats (○) Receiving Both the EGCG Pretreatment and the AFB1 Injection
Chromosome specimens were prepared at various times after 10 mg AFB1/kg body weight was injected i.p. A dose of 60 mg EGCG/kg body weight was orally given 24 hr before the AFB1 injection. Each point represents mean ± S.D. for 6 rats.

Fig. 4. Effect of the Time of EGCG Treatment on the Frequency of Aberrant Cells Induced by AFB1
Chromosome specimens were prepared 18 hr after 10 mg AFB1/kg body weight were injected i.p. A dose of 60 mg EGCG/kg body weight was orally given at various times before the AFB1 injection. Each point represents the mean ± S.D. for 6 rats.

Fig. 3. Relationship between the AFB1 Dose and the Frequency of Aberrant Cells in the Bone Marrow Cells of Rats (●) Receiving only the AFB1 Injection or Rats (○) Receiving Both the EGCG Pretreatment and the AFB1 Injection
Chromosome specimens were prepared 18 hr after various doses of AFB1 were injected i.p. A dose of 60 mg EGCG/kg body weight was orally given 24 hr before the AFB1 injection. Each point represents mean ± S.D. for 6 rats.

Fig. 5. Relationship between the EGCG Dose and the Frequency of Aberrant Cells Induced by AFB1
Chromosome specimens were prepared 18 hr after 10 mg AFB1/kg body weight was injected i.p. EGCG was orally administered in various doses 24 hr before the AFB1 injection. Each point represents mean ± S.D. for 6 rats.
ECG significantly suppressed AFB1-induced CA. The suppressive effect of ECGC was more potent than that of ECG. Only the administration of ECG enhanced them, although there was no significance. On the other hand, in rats given GTP 2 hr before the AFB1 injection, EGC and GA significantly suppressed AFB1-induced CA. The suppressive effect of EGC and GA was the same level. EGCG administered 24 hr before the AFB1 injection induced the most potent suppressive effect under the experimental condition tested.

Cytochrome P450 content and GST activity of the liver of rats treated with EGC or GA 24 hr before sacrifice was the same level as those of rats receiving no-treatment (Table 3).

Rats given 2% green tea infusion as the sole source of drinking water for four days before sacrifice displayed significantly suppressed AFB1-induced CA and this result was consistent with our result on GTE previously reported. However, rats given various kinds of canned tea for four days showed no suppressive effect (Table 4). The amount of GTP in canned tea determined by HPLC analysis was much less than that in 2% green tea infusion (Table 4).

**DISCUSSION**

AFB1, a mycotoxin produced by *Aspergillus flavus*, is a typical natural toxicant in food. This mycotoxin not only has an acute toxic activity but also potent mutagenic and carcinogenic activities and requires metabolic activation via cytochrome P450 enzyme system to exert its biological activities. The major ultimate metabolite of AFB1 is considered to be AFB1-2,3-epoxide. However, it has also been reported that many mutagens and carcinogens act through various free radicals generated during their metabolism. Furthermore, a recent review by Augusto has shown the importance of carbon-centered radicals in the DNA strand breaking.

In the present study, we have investigated the suppressive effect of EGCG, the major polyphenolic
constituent contained in green tea, on AFB₁-induced CA in rat bone marrow cells. The administration of EGCG 24 hr before the AFB₁ injection potently suppressed AFB₁-induced CA. The suppression mechanism of EGCG has not yet been elucidated. We have previously reported that the administration of Sudan III, an inducer of drug-metabolizing enzymes, 24 hr before a carcinogen treatment potently suppressed carcinogen-induced CA, although its administration 2 hr before did not. The suppression of 7,12-dimethylbenz(a)anthracene (DMBA)-induced CA by Sudan III was observed at all periods after the DMBA treatment. It was suggested that the suppressive effect of Sudan III is due to the induction of cytochrome P450 and GST by Sudan III in rat liver. Furthermore, it has been previously reported that glutathione (GSH), ellagic acid and S-methyl methanethiosulfonate (MMTS), not an inducer of drug-metabolizing enzymes, significantly suppressed carcinogen-induced CA, but their suppressive effect was maximum when given 2 hr or 30 min before the carcinogen treatment. The suppression of AFB₁- or methyl methanesulphonate (MMS)-induced CA by MMTS was also observed at all periods after the MMS treatment. Moreover, it was suggested that the suppression of carcinogen-induced CA by GSH or ellagic acid results from the direct reaction with the active metabolite of carcinogen and that the suppression by MMTS results from the ability of MMTS to modify –SH group in proteins.

The suppression of AFB₁-induced CA by EGCG was observed only at the late period (18, 24 and 48 hr) after the AFB₁ injection. This result is different from the previous result showing that Sudan III and MMTS suppressed carcinogen-induced CA at all periods after the carcinogen treatment. Figure 2 seems to indicate that the peak in the percentage of aberrant cells induced by AFB₁, shifted from 18 hr to 12 hr by the administration of EGCG. Therefore, this result may suggest that the administration of EGCG accelerates the rate of AFB₁ metabolism in rat liver and that the decrease of active metabolites of AFB₁ reaching target cells produces the suppression of AFB₁-induced CA at the late period after the AFB₁ injection. However, the administration of EGCG 24 hr before sacrifice did not result in a significant increase in cytochrome P450 content or GST activity (Table 3).

CA induced by DMBA, which needs metabolic activation as well as AFB₁, were also suppressed by EGCG given 24 hr before the DMBA treatment, but CA induced by direct-acting carcinogen such as MMS and n-butyl-N-nitrosourea were not suppressed by EGCG (unpublished data). Therefore, the suppression by EGCG seems to be due not to the direct action upon the carcinogen but to the indirect action through the intermediation of the microsomal enzyme system, such as the modification of cytochrome P450 by EGCG. This suggestion is supported by the following reports. Qin et al. have reported that the pretreatment of rats with 0.5% green tea in their drinking water for 2 or 4 weeks did not produce a significant increase in cytochrome P450 content but enhanced microsome-mediated formation of non-toxic hydroxylated metabolites of AFB₁ by 2–3 fold. It has also been reported by Sohn et al. and Bu-Abbas et al. that rats administered GTE for 4 or 6 weeks displayed no significant increase in total cytochrome P450 content in the liver but did display a significant increase in O-dealkylase activity of ethoxyresorufin (CYP1A1), methoxyresorufin (CYP1A2) and pentoxyresorufin (CYP2B1).

Table 2 indicates the suppressive effect of individual GTP on AFB₁-induced CA. In rats given GTP 24 hr before the AFB₁ injection, EGCG and
ECG significantly suppressed AFB1-induced CA and the suppressive effect of individual GTP was in the order: EGCG > ECG > GA > EGC > C > EC. This order was nearly the same as the order of their biochemical activities, such as the antioxidative activity, bactericidal activity or the inhibition of P450, which were previously reported by other researchers. The suppressive effect of GTP on AFB1-induced CA was in proportion to the number of OH groups present in GTP, except GA. Therefore, the number of OH groups and the gallic acid moiety present in GTP seems to be important for the suppressive effect of GTP given 24 hr before the AFB1 injection. On the other hand, in rats given GTP 2 hr before the AFB1 injection, EGC and GA significantly suppressed AFB1-induced CA and the suppressive effect of individual GTP was in the order: GA = EGC > EC > C > ECG > EGCG. Their suppressive effect was in inverse proportion to the number of OH groups present in GTP, except EGC. Therefore, our results mentioned above may suggest that the suppression mechanism of GTP has two different ways depending on the administration time and the kind of GTP.

Kada53) divided antimutagens into desmutagen and bio-antimutagen according to their modes of action. The former inactivates or destroys mutagens directly or indirectly outside the cell and the latter suppresses the process of mutagenesis itself inside the cell. The present results may show that EGCG suppresses carcinogen-induced CA in a desmutagenic manner because the administration of EGCG to rats previously exposed to AFB1 showed no suppression (data not shown). Jain et al.13 also reported that GTE and GTP such as EC, ECG and EGC decreased the mutagenic activity of N-methyl-N′-nitro-N-nitrosoguanidine to E. coli WP2 in vitro in a desmutagenic manner. However, Kada et al.53 reported that EGCG has a bio-antimutagenic activity, that is, EGCG improves the fidelity of DNA replication. Sasaki et al.54,55 and Shimoi et al.56 also reported that tannic acid obtained by hydrolysis from gallotannin is a bio-antimutagen that enhances the excision repair activity.

The number of young people who often drink canned tea is gradually increasing in Japan because it is convenient and portable. However, the present study indicates that rats given 2% green tea infusion for four days displayed significantly suppressed AFB1-induced CA but rats given various kinds of canned tea showed no suppressive effects. The reason canned tea gave no suppression may be explained by the limited amount of GTP present in canned tea. Therefore, we had better drink green tea that is made by brewing green tea leaves with boiling water just before drinking from the point of view of chemoprevention against cancer.

The present study is consistent with epidemiological studies5–9 suggesting that the habitual drinking of green tea decreases the incidence of certain types of cancer. However, some serious problems, such as the toxicity of EGCG or its metabolites, remain in considering them as a cancer chemopreventive agent. Especially their metabolism has not yet been elucidated at all. Further studies are needed for clarification.

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