Synergistic effects of (-)-epigallocatechin gallate with sulindac against colon carcinogenesis of rats treated with azoxymethane.

Tomokazu Ohishi1, Yosuke Kishimoto2, Yukihiko Hara3, Junichi Hasegawa3, Mamoru Isemura1

1: School of Food and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan
2: Department of Clinical Pharmacology, Faculty of Medicine, Tottori University, 86 Nishicho, Yonago, Tottori 683-8503, Japan
3: Central Research Laboratories, Tokyo Food Techno Company, Ltd., Miyahara 223-1, Fujieda, Shizuoka 426-0133, Japan

Summary

(-)-Epigallocatechin gallate (EGCG), a major constituent of green tea, has been shown to exhibit anti-cancer activity. Sulindac is also known as a cancer preventive agent against colon cancer, but its usage is restricted because of its adverse effects, as exemplified by gastrointestinal bleeding. In the present study, we examined whether a combination of EGCG and sulindac shows synergistic effects for cancer-preventive activity for rat colon carcinogenesis induced by azoxymethane (AOM); we examined the number of aberrant crypt foci (ACF) representing preneoplastic lesions, the argyrophilic nucleolar organizer region (AgNOR) as an indicator of cell proliferation, and the incidence of apoptosis. The results of the AgNOR analysis indicated that the treatment with EGCG and/or sulindac suppressed AOM-induced cell proliferation. The present results also revealed that the combination of EGCG and sulindac synergistically enhanced apoptosis significantly (p<0.01). Thus, our findings suggest that EGCG with sulindac synergistically suppresses ACF formation by enhancing apoptosis and, therefore, that EGCG is a suitable candidate for use in combination with cancer-preventive agents such as sulindac to reduce their adverse effects.

Key words

(-)-Epigallocatechin gallate, sulindac, apoptosis, colon, rat

Introduction

Green tea has been consumed in many countries throughout the world without severe adverse effects. The biological responses of green tea are believed to be mediated by its main polyphenol (-)-epigallocatechin gallate (EGCG) and EGCG has been shown to possess superior chemopreventive effects[1-5]. Recently, Suganuma et al. [6] reported that the anti-tumor effects of EGCG were synergistically enhanced in vitro by sulindac, a non-steroidal anti-inflammatory drug (NSAID), in a lung cancer cell line. Furthermore, a green tea extract and sulindac synergistically reduced the number of tumor incidence and the tumor size in multiple intestinal neoplasia mice[7]; however, the anti-tumor mechanism remains to be explored.

Numerous studies from animal models have accumulated evidence to suggest that the administration of NSAIDs is a potentially viable option in the chemoprevention of colon carcinogenesis induced by azoxymethane (AOM) in rats[8-11]. Sulindac can be used as a cancer preventive agent for colon cancer including familial adenomatous polyposis[12], but the usage of this drug is restricted because of its adverse effects, as exemplified by gastrointestinal bleeding after the long administration. For this reason, it has been requested to reduce the amount of this drug and/or increase the preventive effect against colon cancer.

In the present study, we studied the synergistic effects of EGCG with sulindac for cancer-prevention activity using an AOM-induced rat colon carcinogenesis model.

Materials and methods

Chemicals

EGCG with purity of more than 95% was obtained from Mitsui Norin Co., Ltd. (Shizuoka, Japan). AOM and sulindac (cis-5-fluoro-2-methyl-1-[p-(methyl-sulphinyl) benzylidene] indene-3-acetic acid) were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

Five-week-old male F344 rats purchased from SLC (Shizuoka, Japan) were quarantined for one week and allocated randomly to experimental or control groups. Ethics approval for the study was obtained from the Committee for Animal Experimentation of Tottori University.
A total of 90 male rats were divided into seven groups. Starting at six weeks of age, rats in groups 2, 3, 4 and 5 were subcutaneously injected AOM in sterile saline at a dose of 15 mg/kg body weight once a week for three weeks. Group 2 received AOM alone. Groups 3, 4 and 6 were administered 10 mg/kg body weight sulindac in 5% gum arabic aqueous solution by oral gavage three times a week (Monday, Wednesday, and Friday) during the experiment. Groups 3, 5 and 7 were administered 0.01% EGCG dissolved in water for drinking during the experiment. The EGCG solution was prepared daily and kept in a black bottle to minimize light-caused degradation. Group 1 did not receive any agent (AOM, sulindac or EGCG) throughout the study and served as control. Animals in group 1 were administered subcutaneous injections of sterile saline instead of AOM. Each group was subdivided into two subgroups: one for counting the number of ACF and the other for apoptosis and argyrophilic nucleolar organizer region (AgNOR) stainings. All rats were sacrificed at 10 weeks of age and the colons removed, flushed with saline, and opened from the anus to the caecum. For counting the number of ACF, the opened colon was fixed flat on a paper filter in 10% buffered formalin for 24 h.

Identification of ACF

Fixed colons were stained with 0.5% methylene blue in saline. The number of ACF/colon and the number of aberrant crypts in each focus were determined microscopically at ×40 magnification, and recorded as described previously [13,14]. Crypt multiplicity was defined as the number of aberrant crypts in each focus, categorized as either one, two, three, or four and more aberrant crypts per focus. The scores were checked by two observers in a double blind manner.

AgNORs staining

Staining for AgNORs was done to estimate cell proliferation using as described previously [15]. The normally appearing specimens were formalin-fixed and paraffin-embedded, four-μm-thick sections were prepared, and the stained sections were examined using a microscope at a ×100 magnification. In each specimen, more than 200 cells were selected randomly, and the separable AgNOR dots with a diameter of more than 1 mm were counted. Those with diameters less than 1 mm were not counted because of the possibility of non-specific precipitation and background staining.

Detection of apoptosis

To assess the frequency of apoptosis, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method was performed following the protocol provided by the manufacturer (Apoptosis In Situ Detection Kit; Wako Pure Chemical Industries, Ltd.). In brief, after deparaffinization of formalin-fixed paraffin-embedded tissues, nuclei of tissue sections were stripped from proteins by incubated with 20 mg/ml proteinase K for 5 min at 37°C. The sections were incubated for TUNEL TdT buffer for 5 min at 37°C. Endogenous peroxidase was inactivated by covering the sections with 3% H2O2 for 5 min at room temperature, and then stained with peroxidase-conjugated avidin for 10 min at 37°C. After staining with a diaminobenzidine solution, the sections counter-stained with methyl green were examined under a microscope at ×400 magnification.

Statistical analysis

In each group, the numbers of ACF and AgNOR, and the frequency of apoptosis were expressed as the mean±SD, and differences between groups were analyzed using Student’s t test, where p<0.05 was considered significant.

Results and Discussion

The number of ACF observed in each experimental group is listed in Table 1. Crypt multiplicity was also counted. In rats of group 2, the AOM treatment induced an average of 46.2±4.9 ACF/colon and 3.1±2.0 foci containing multiple (≥4) aberrant crypts/focus. Administration of sulindac (group 4) or EGCG (group 5) significantly reduced the incidence of ACF/colon and the number of aberrant crypts/foci. The number of ACF in group 3 was further reduced to 10.0±3.2, suggesting synergistic effects of EGCG with sulindac. Thus, the data showed that EGCG suppressed ACF formation and synergistically reduced the development of ACF when used in combination with sulindac.

AgNORs were examined to estimate the level of cell proliferation. As shown in Table 2, the mean number of AgNORs/nucleus in group 2 was greater than those of the other groups. The number of
Table 1  Effects of sulindac and EGCG on AOM-induced ACF in the rat colon

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence</th>
<th>No. of ACF/colon</th>
<th>No. of foci containing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 crypt</td>
</tr>
<tr>
<td>1. No treatment</td>
<td>0/7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. AOM alone</td>
<td>7/7</td>
<td>46.2±4.9</td>
<td>13.5±4.8</td>
</tr>
<tr>
<td>3. AOM+sulindac+EGCG</td>
<td>5/5</td>
<td>10.0±3.2</td>
<td>3.2±1.1</td>
</tr>
<tr>
<td>4. AOM+sulindac</td>
<td>7/7</td>
<td>21.4±3.4</td>
<td>5.6±0.9</td>
</tr>
<tr>
<td>5. AOM+EGCG</td>
<td>6/6</td>
<td>19.5±5.8</td>
<td>7.2±3.9</td>
</tr>
<tr>
<td>6. Sulindac alone</td>
<td>0/7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7. EGCG alone</td>
<td>0/5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Number of rat colons with ACF/total number of colons scored.
b Mean±SD.
c,d Significantly different from group 2 by Student's t test (p<0.05 and p<0.01).
d Significantly different from group 4 and 5 by Student's t test (p<0.01).

Table 2  Incidence of apoptosis and AgNORs count of the colonic epithelium of rats in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats examined</th>
<th>AgNORs count/nucleus</th>
<th>Incidence of apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.No treatment</td>
<td>8</td>
<td>1.15±0.59</td>
<td>0.019±0.005</td>
</tr>
<tr>
<td>2.AOM alone</td>
<td>9</td>
<td>1.81±0.005</td>
<td>0.033±0.005</td>
</tr>
<tr>
<td>3.AOM+Sulindac+EGCG</td>
<td>7</td>
<td>1.21±0.08</td>
<td>0.061±0.019</td>
</tr>
<tr>
<td>4.AOM+Sulindac</td>
<td>5</td>
<td>1.21±0.69</td>
<td>0.043±0.019</td>
</tr>
<tr>
<td>5.AOM+EGCG</td>
<td>5</td>
<td>1.30±0.08</td>
<td>0.041±0.013</td>
</tr>
<tr>
<td>6.Sulindac alone</td>
<td>5</td>
<td>1.17±0.67</td>
<td>0.012±0.001</td>
</tr>
<tr>
<td>7. EGCG alone</td>
<td>6</td>
<td>1.13±0.04</td>
<td>0.015±0.009</td>
</tr>
</tbody>
</table>

a Mean±SD.
b Significantly different from groups 2-7 by Student's t test (p<0.01).
c Significantly different from groups 5-7 by Student's t test (p<0.01).
d Significantly different from group 1, 3 and 4 by Student's t test (p<0.01).

AgNORs/nucleus was significantly decreased in the group of rats treated with sulindac, EGCG or both (groups 3, 4 and 5) compared to that of group 2 treated with AOM alone. These findings indicated that treatment with sulindac or EGCG reduced the cell proliferation of the colonic mucosa. The incidences of apoptosis in groups 2-5 were significantly elevated as compared to control group 1 (Table 2). In group 2 treated with AOM alone, the average incidence of apoptosis was about two fold of the control value. This finding may suggest that apoptosis was induced to exclude the cells damaged by the AOM treatment. In groups 4 and 5, apoptosis indices were higher than that of group 2, suggesting that sulindac and EGCG enhanced induction or apoptosis, presumably to eliminate damaged cells. The
value for group 3 was even higher than those for groups 4 and 5, suggesting that EGCG enhanced the apoptosis synergistically with sulindac.

In summary, our findings suggest that EGCG with sulindac synergistically suppresses ACF formation by enhancing apoptosis and, therefore, that EGCG is a suitable candidate for use in combination with cancer-preventive agents such as sulindac to reduce there adverse effects.

References