

Inhibition of *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine-induced Carcinogenesis by (-)-Epigallocatechin Gallate in the Rat Glandular Stomach¹

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ABSTRACT

Recently, an epidemiological study showed a lower risk of gastric cancer among people who consume a large amount of green tea. (-)-Epigallocatechin gallate (EGCG), one of the main constituents of green tea, inhibited tumor promotion by teleocidin in a two-stage carcinogenesis experiment with the use of mouse skin.

The inhibitory effect of EGCG on *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced carcinogenesis of the glandular stomach in rats was examined. The percentage of tumor-bearing rats in the group treated with MNNG plus EGCG was 31%, compared to 62% in the MNNG group. The difference was statistically significant ($P < 0.05$).

To assess the effect of p.o. administration of EGCG, the gastric mucosal cellular kinetics was examined with the use of the bromodeoxyuridine labeling index, ornithine decarboxylase activity, and tissue polyamine levels. The labeling index of the EGCG treatment group decreased significantly ($P < 0.05$) compared to the EGCG plus MNNG treatment group. The ornithine decarboxylase activity and tissue spermidine levels were also decreased. On the other hand, the tissue putrescine and spermine levels were partly increased. These findings suggest that EGCG inhibits the cellular kinetics of the gastric mucosa during the promotion stage of MNNG-induced gastric carcinogenesis. EGCG may be useful in preventing gastric carcinogenesis. Moreover, EGCG may be applied clinically without any harmful effects and at a low cost.

INTRODUCTION

Prevention of carcinogenesis is one of the major strategies for cancer control. The inhibitory effects of several preventive agents in experimental carcinogenesis have been reported. However, some of these agents have harmful effects and are usually expensive. We considered that the inhibitory effect of a natural product in the diet is safer and can be obtained easily at a low cost. Recently, an epidemiological study showed a lower risk of gastric cancer among people who consume a large amount of green tea (1). An experiment on two-stage skin carcinogenesis in mice showed that EGCG,³ one of the main constituents of green tea, is an effective antitumor promoter (2). Previously, we also reported that EGCG inhibited the incidence of tumor in mouse duodenal carcinogenesis induced by *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine, supporting its previously reported effect on carcinogenesis in the alimentary tract. In this study, the inhibitory effect of EGCG was examined in MNNG-induced carcinogenesis of the glandular stomach in the rats (3). The effect of the p.o. administration of EGCG on gastric mucosal cellular kinetics was examined with the use of BrdU immunohistochemical staining. ODC activity and tissue polyamine levels were also measured.

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³ The abbreviations used are: EGCG, (-)-epigallocatechin gallate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; ODC, ornithine decarboxylase; BrdU, bromodeoxy-uridine.

MATERIALS AND METHODS

Materials. This study was carried out according to the experimental protocol shown in Fig. 1. Ninety 8-week-old male Wistar/KOB rats, (Ishikawa Experimental Instruments, Maebashi, Japan) were given free access to drinking water and standard laboratory chow MF (Oriental Yeast Co., Tokyo, Japan). EGCG was purchased from the National Cancer Center Institute (Tokyo, Japan) and isolated from Japanese green tea leaves (*Camellia sinensis*). The product contained 85% EGCG, 10% (-)-epicatechin, and 5% (-)-epicatechin gallate as reported previously (1).

Carcinogenesis. The rats were then divided into four groups: control; EGCG treatment; MNNG treatment; and MNNG plus EGCG treatment groups. MNNG (Nakalai Tesque Co., Kyoto, Japan) was given p.o. as a solution at a concentration of 80 mg/liter for the first 28 weeks. The experimental groups were given a solution of 0.05% EGCG p.o. for 15 weeks, and the control group was given tap water. Then, all rats were killed at the 44th week of the experiment. The esophagus, forestomach, glandular stomach, and duodenum were removed. The stomach was opened along the greater curvature, stretched out flat on a cork board, and fixed in 70% ethanol for 2 h. After fixation, the gastric mucosa was examined grossly. The location, shape, size, and number of tumors were recorded. The tumor and normal appearing gastric mucosa were then fixed in 10% formalin, sectioned serially into 3 pieces along the lesser curvature of the stomach, and embedded for histological examination.

BrdU Staining. The cellular kinetics of these glandular stomach specimens was studied with the use of immunohistochemical staining with BrdU (Takeda Pharmaceutical Co., Osaka, Japan). One h before the death of the rats, 20 mg/kg of BrdU was injected i.p. Immunohistochemical staining by the ABC method was used to detect the developmental stage of the cells. BrdU was taken up by the DNA-developing stage cells, staining them brown. The percentage of stained cells at the fundic gland was calculated and expressed as labeling index (4-6). The relationship between the value of the labeling index and the histological features of the gastric tumors was estimated.

ODC Activity. ODC activity, a useful indicator of the promotion step of carcinogenesis, was measured at the 44th week of the experiment. All gastric mucosal samples were frozen immediately with liquid nitrogen and stored at -80°C until analysis for ODC activity, which was based on the method of Russel and Snyder (7). Tissue samples of about 100 mg were homogenized in 1.0 ml of 50 mM sodium phosphate buffer (pH 7.2), containing 0.1 mM pyridoxal phosphate and 0.1 mM EDTA. Insoluble material was removed by centrifugation at 30,000 × g for 20 min, and the protein content of the supernatant was determined by the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA) with the use of BSA as a standard. ODC activity was determined by measuring the release of radiolabeled CO₂. The incubation mixture (final volume, 2 ml) in Warburg flasks consisted of 0.4-ml tissue extracts containing approximately 1.0 mg of protein. After preincubation at 37°C for 10 min, 0.2 ml of a solution containing 18.5 kBq DL-[1-¹⁴C]ornithine monohydrochloride (1.954 GBq/mmol, DuPont New England Nuclear, Boston MA) and 0.2 μmol L-ornithine were added. Incubation was carried out for 60 min at 37°C. The reaction was trapped by the addition of 0.8 ml of 2 M citric acid, and the released CO₂ was trapped in 0.2 ml of Protosol. This assay was done in duplicate. One enzyme unit is defined as 1 pmol CO₂/h/mg protein.

Tissue Polyamine. Tissue specimen was obtained from the glandular stomach at the greater curvature of the antrum, frozen immediately with liquid nitrogen, and stored at -80°C until measurement of the tissue polyamine level. The supernatant of the tissue specimens was prepared by the same method as that for the measurement of ODC activity as above mentioned. Then, the protein content of the supernatant was determined by the Bio-Rad protein assay kit. Putrescine, spermidine, and spermine in the supernatant were measured by the conventional ion-pairing reversed phase HPLC method (8).

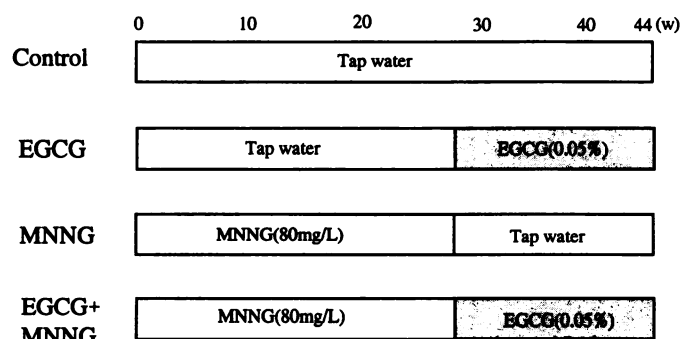


Fig. 1. Experimental schedule of gastric carcinogenesis of MNNG and EGCG. MNNG was given p.o. as a solution at a concentration of 80 mg/liter (L) for the first 28 weeks (w). A solution of 0.05% EGCG was given p.o. for 15 weeks.

Statistics. The significance of differences in tumor incidence was analyzed with the use of the χ^2 test, and other data were analyzed with the use of the Mann-Whitney test.

RESULTS

During the experiment, the rats in the 2 groups did not show any significant difference in body weight (Fig. 2). The inhibitory effects of EGCG were examined in terms of the number of tumors, tumor incidence (percentage of tumor-bearing rats), average number of tumors/rat, and mean diameter of tumors. The percentage of tumor-bearing rats in the group treated with MNNG plus EGCG was 31%, compared to the 62% in the control group (Table 1). The differences in both tumor incidence and average number of tumors/rat were statistically significant ($P < 0.05$). Rats that did not receive MNNG treatment (*i.e.*, control group and EGCG treatment group) had no evidence of gastric tumors.

Histologically, the gastric tumors consisted of adenocarcinoma, adenoma and hyperplasia in the glandular stomach, and papilloma at the border between the forestomach and the glandular stomach. In the MNNG treatment group, 5 adenocarcinomas, 5 adenomas, 18 hyperplasias, and 2 papillomas were seen. On the other hand, 2 adenocarcinomas, 2 adenomas, and 11 hyperplasias were seen in the MNNG plus EGCG treatment group (Table 2). Every type of gastric tumors was decreased with the administration of EGCG.

Fig. 3 shows the results of the cellular kinetic study with BrdU immunohistochemical staining. The labeling index in the MNNG treatment group was $20.8 \pm 3.0\%$ (SE), compared to $15.3 \pm 3.4\%$ in the MNNG plus EGCG treatment group. The difference in the labeling index between these two groups was statistically significant ($P < 0.05$). However, no definite relationship was found between the labeling index and the histological features of carcinogenesis. This suggests that EGCG inhibits the cellular kinetics of the gastric mucosa during the promotion stage of MNNG-induced gastric carcinogenesis.

ODC activity in the MNNG treatment group was 12.0 ± 3.5 pmol $\text{CO}_2/\text{h}/\text{mg}$ protein compared to the 10.7 ± 3.5 in the MNNG plus EGCG treatment group (Fig. 4). The level of ODC activity decreased with the administration of EGCG, but the difference was not statistically significant. No definite relationship was found between ODC activity and the histological features of carcinogenesis.

The values of putrescine in the MNNG and MNNG plus EGCG treatment groups were 33.4 ± 4.2 (SE) and 44.6 ± 5.2 , respectively. The values of spermine in the MNNG and MNNG plus EGCG treatment groups were 1.22 ± 0.06 and 1.25 ± 0.07 , respectively. The values of spermidine in the MNNG and MNNG plus EGCG treatment groups were 1.45 ± 0.10 and 1.22 ± 0.05 , respectively. The increase in the tissue spermine and putrescine levels was not significantly

different between the MNNG treatment group and MNNG plus EGCG treatment group. The tissue spermidine level was significantly ($P < 0.05$) decreased by the EGCG treatment (Fig. 5).

DISCUSSION

Green tea contains many polyphenolic constituents, including tannic acid, which is similar in chemical structure to EGCG. The effects of tannic acid in green tea have been reported to be protection against tumor development, suppression of the genotoxicity of carcinogens, and inhibition of lipid oxidation by free radicals that attack DNA (9). Tannic acid among the naturally occurring plant phenols has been reported to inhibit skin tumorigenesis in mice (10). EGCG inhibited 7,12-dimethylbenz[*a*]anthracene- and 12-*o*-tetradecanoyl-phorbol-13-acetate-induced two-stage skin carcinogenesis in the mice. EGCG also inhibited the promotive effect of teleocidin, which suggests that it is an effective antitumor promoter (2). Several experimental studies revealed that green tea polyphenols and EGCG inhibited the tumor incidence of chemical carcinogenesis in the duodenum (11), colon (12, 13), skin (14–16), and lungs (17, 18).

In the epidemiological study of the Japanese, pickles and salty foods were associated with the risk of gastric cancer (19). On the other hand, vegetables and fruits have also been reported to be a low risk diet for gastric cancer (20). These diets contain plant polyphenols. Recently, a case-control study of gastric cancer and diet reported that the risk of gastric cancer was decreased among those with a high consumption of green tea (10 or more cups per day) (1). However, the risk of colorectal cancer was similar in the two groups with high and low consumption of green tea (21). One reason for the protective effect of green tea against gastric cancer may be due to the high content of vitamin C. The concentration of EGCG in green tea was about 18%. EGCG in 10 cups of green tea corresponds to about 200 mg of EGCG. In this experiment, 0.05% EGCG was administered in drinking water. This concentration was consistent with the consumption of 1 g/day of EGCG in humans.

The metabolism of EGCG is not well understood. The mechanism of the protective effect of EGCG against cancer is still not well defined. EGCG has a potent antioxidative effect (22) and blocks the interaction of tumor promoters, hormones, and growth factors with their receptors (23). To examine the role and mechanism of EGCG, we used the BrdU-staining assay and measurement of ODC activity and tissue polyamine levels. Immunohistochemical staining with BrdU was used to assess the cell kinetics of the gastric mucosa (4–6, 24). Cell proliferation in each experimental group was compared by the use of the labeling index. The labeling index in the MNNG plus EGCG treatment group was significantly lower than that in the

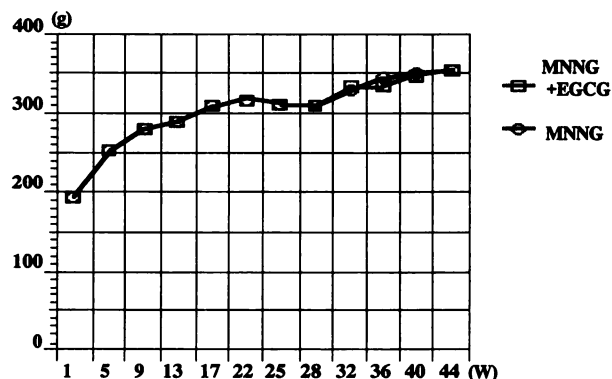


Fig. 2. Body weight changes of gastric carcinogenesis given MNNG and EGCG. The difference between the two groups was not statistically significant in any period of the experiment.

Table 1 Incidence and size of tumors in the glandular stomach in rats

	Control	EGCG	MNNG	MNNG + EGCG
No. of tumors	0	0	30	15
Tumor incidence (%)	0/10 (0%)	0/10 (0%)	21/34 (62%) ^a	11/35 (31%) ^a
Tumors/rat (mean ± SD)	0	0	0.88 ± 0.87 ^a	0.43 ± 0.71 ^a
Diameter of tumors (in mm; mean ± SD)			2.25 ± 2.15	2.67 ± 2.34

^a $P < 0.05$.Table 2 Histological findings of tumors in the glandular stomach in rats^a

Histology	MNNG	MNNG + EGCG
Adenocarcinoma	5/34	2/35
Adenoma	5/34	2/35
Adenomatous hyperplasia	18/34	11/35
Papilloma	2/34	0/35

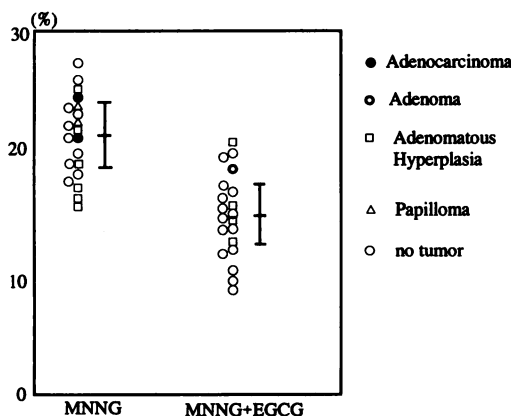
^a The differences between the two groups were not significant.

Fig. 3. Labeling index in MNNG-induced rat gastric carcinogenesis. In both the MNNG group and MNNG + EGCG group, the labeling index of gastric mucosa was selected in 20 random specimens. Bars, mean ± SD. The difference between the two groups was statistically significant ($P < 0.05$).

MNNG treatment group. This suggests that EGCG inhibits the cell kinetics of the gastric mucosa during the promotion stage of MNNG-induced gastric carcinogenesis and has an antipromotive effect.

ODC is a rate-limiting enzyme of polyamine biosynthesis, and the level of ODC is elevated at the time of acceleration of cell proliferation and development (25, 26). ODC activity is well known as a useful indicator at the promotion step of carcinogenesis (27). In our study, ODC activity was slightly depressed by the administration of EGCG. EGCG may inhibit cell proliferation and development at the promotion step of MNNG-induced gastric carcinogenesis in rats. To elucidate this phenomenon, ODC activity must be examined.

In the ornithine biosynthesis pathway, putrescine, spermine, and spermidine were synthesized from ornithine by the enzyme activity of ODC. The tissue level of spermine, the final product of polyamine biosynthesis, and tissue putrescine and spermine levels were not changed significantly, despite the decrease of ODC activity. The tissue spermidine level might decrease significantly with the decrease of cell proliferation under the control of ODC. ODC and polyamine levels might change much earlier during the carcinogenesis process, and measurement by these assays over the time course of carcinogenesis might be helpful.

Histological studies of MNNG-induced gastric glandular tumorigenesis indicate that EGCG prevents gastric mucosal development or stops its progression. It is necessary to identify and develop biomarkers that correlate with the appearance and regression of intraepithelial neoplasia (28).

EGCG is a component in green tea. Green tea is a popular beverage in Japan. Green tea lovers drink 10 or more cups of green tea daily, which contains 200 mg/day or more of EGCG. It is expensive to purify the EGCG from green tea, because green tea contains components similar to EGCG. The crude green tea extract may be useful for the clinical application for chemoprevention (29). In fact, the green tea extract has been used as a food additive for the prevention of tooth decay in Japan. It is safe without any harmful effect. The epidemiological study of green tea consumption and several experimental studies about the inhibition of carcinogenesis with green tea and its component strongly suggest its efficacy in the clinical chemoprevention trial with green tea (30).

In conclusion, EGCG inhibited the MNNG-induced carcinogenesis of the glandular stomach in rats. The cellular kinetics of the gastric mucosa with BrdU showed a significant decrease in the labeling index

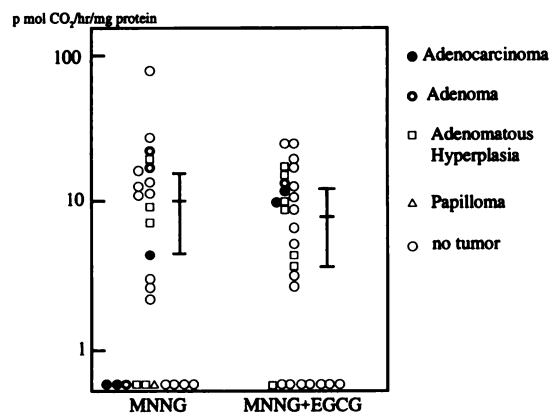


Fig. 4. ODC activity in MNNG-induced rat gastric carcinogenesis. In the MNNG group and MNNG + EGCG group, ODC activity of gastric mucosa was selected in 26 and 28 random specimens, respectively. Bars, mean ± SD. The difference between the two groups was not statistically significant.

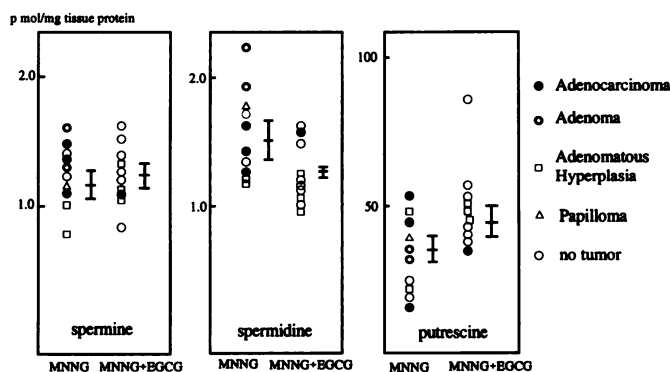


Fig. 5. Tissue polyamine levels in MNNG-induced rat glandular stomach. In the MNNG group and MNNG + EGCG group, gastric mucosa were selected in 10 random specimens each, and the tissue spermine, spermidine, and putrescine levels were measured. Bars, mean ± SD. The difference of tissue spermine and putrescine levels between the two groups was not statistically significant. The difference in the tissue spermidine level was statistically significant ($P < 0.05$).

with the use of EGCG. These findings suggest that EGCG is useful in preventing gastric carcinogenesis. Moreover, clinical application of EGCG without any harmful effects and at a low cost is awaited.

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