SUPPRESSIVE EFFECTS OF CHLOROGENIC ACID ON N-METHYL-N-NITROSOUREA-INDUCED GLANDULAR STOMACH CARCINOGENESIS IN MALE F344 RATS

Masahito SHIMIZU1,2, Naoki YOSHIMI1, Yasuhiro YAMADA1, Kengo MATSUNAGA1, Kunihiro KAWABATA1, Akira HARA1, Hisataka MORIWAKI1 and Hideki MORI1

1Department of Pathology and 2 1st Department of Internal Medicine, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500-8705, Japan

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ABSTRACT — The modifying effects of chlorogenic acid (CA) on N-methyl-N-nitrosourea (MNU)-induced glandular stomach carcinogenesis were investigated in five groups of male F344 rats. Rats in Groups 1 through 3 were given MNU in drinking water at a concentration of 400 ppm for 12 weeks. Animals of Group 1 were then kept on the basal diet alone, and those of Group 2 or 3 were fed a diet containing 500 or 250 ppm CA for a subsequent 22 weeks. Group 4 was exposed to CA alone through the experimental period (36 weeks), and Group 5 was given the basal diet continuously and treated as a control. At the end of the experiment, the incidence of glandular stomach carcinoma of Group 3 was significantly smaller than that of Group 1 (p<0.03). The incidence of adenomatous hyperplasia of Group 2 was also significantly lower than that of Group 1 (p<0.02). In addition, the proliferating cell nuclear antigen (PCNA) labeling index of the epithelial cells from the non-neoplastic mucosa in rats of Group 2 or 3 was significantly smaller than that of Group 1 (p<0.0001). These results suggest that CA has a chemopreventive effect on MNU-induced rat glandular stomach carcinogenesis by exposure during the post-initiation phase, and CA may be a promising agent for prevention of human stomach cancer.

KEY WORDS: Chlorogenic acid, Chemoprevention, Glandular stomach carcinogenesis, MNU, Rats

INTRODUCTION

Stomach cancer is one of the most prevalent malignancies in Japan, and dietary habits have been regarded as an important etiological factor (Tajima and Tominaga, 1985). On the other hand, there is an inverse relationship between gastric cancer risk and intake of raw vegetables and fruits (Boeving, 1991). It is known that plant phenolics are present in commonly consumed foods (Brown, 1980) and some of them have antmutagenic and anticaninecarcinogenic activities (Newmar, 1987). Chlorogenic acid (CA) is a member of the plant phenolics, with higher concentrations in potatoes, broccoli and coffee beans (Al-Saikhan et al., 1995; Sondheimer, 1958). We have shown the chemopreventive effects of CA on digestive organ carcinogenesis by methylazoxymethanol-acetate and 4-nitroquinoline-1-oxide in rodents (Mori et al., 1986; Tanaka et al., 1993). Although there are accumulated data showing chemopreventive effects of plant-derived chemicals including phenolics in digestive organ carcinogenesis (Mori et al., 1995; Mori et al., 1997), relatively little information on stomach carcinogenesis has been reported (Tanaka et al., 1995; Tatsuta et al., 1983). Application of N-methyl-N-nitrosourea (MNU) is an experimental model for selective induction of stomach cancer in the antral mucosa of rats and mice (Hirota et al., 1987; Tatematsu et al., 1993). Previously, our group has proved that a naturally occurring phenolic acid, protocatechuic acid, inhibits MNU-induced glandular stomach carcinogenesis (Tanaka et al., 1995). In this study, the chemopreventive potential of CA for stomach carcinogenesis was examined in the rat carcinogenesis model with MNU.

Meanwhile, cell proliferation has been considered...
to play an important role in carcinogenic processes (Mori et al., 1996). Proliferating cell nuclear antigen (PCNA) immunohistochemistry is regarded as a useful biomarker for cell-proliferative activity in stomach carcinogenesis (Kaminishi et al., 1996). Presently, we also measured the PCNA labeling index of the mucosal epithelium in this model for carcinogenesis.

MATERIALS AND METHODS

Animals, diets and chemicals

Male F344 rats 5 weeks old were purchased from Japan SLC, Inc. (Hamamatsu). MNU was obtained from Sigma Chemical Co. (St. Louis, MO, U. S. A.). CA was purchased from Tokyo Kasei Chemical Co. (Tokyo). Basal diet, CE-2 was obtained from CLEA Japan, Inc. (Tokyo).

Treatment of Animals

After quarantine for 1 week, 78 rats were divided into 5 groups and kept in a room controlled at 23 ± 2°C and 50% ± 10% humidity on a 12hr light/dark cycle. Groups 1 (20 rats), 2 (21 rats) and 3 (17 rats) were given 400 ppm MNU in the drinking water in light-shielded bottles for 12 weeks. MNU was dissolved in distilled water and freshly prepared 3 times a week. Groups 2 and 3 were given 500 and 250 ppm, respectively, of CA mixed in powdered CE-2 diets for 22 weeks, starting 1 week after the end of MNU treatment. Group 4 (10 rats) was fed a diet containing 500 ppm CA alone throughout the experiment. Group 5 (10 rats) was kept on the basal diet during the experiment (Fig. 1).

At the end of the experiment (36 weeks after the start), all rats were sacrificed, stomachs together with duodenum were removed and opened along the greater curvature, and the stomach mucosa was carefully examined. The resected specimens were fixed flat on a paper filter in 10% buffered formalin for 24 hr, after which tissues were cut for 4-mm-wide strips parallel to the lesser curvature, embedded in paraffin, cut into 2 sections (4-μm), and one stained with hematoxylin and eosin for histopathological examination. Neoplastic lesions were classified as adenocarcinomas and adenomatous hyperplasias. Adenocarcinomas demonstrated severe cellular atypia and adenomatous hyperplasia consisting of excessive glandular proliferation with little or no cellular atypia, being regarded as precancerous lesions for gastric carcinomas (Tatematsu et al., 1993) (Photo 1 A, B).

PCNA immunohistochemistry

Another section was subjected to immunohisto-
Inhibition of glandular stomach carcinogenesis by chlorogenic acid.

Photo 1. (A) Photomicrograph of adenomatous hyperplasia in the glandular stomach of a rat in Group 1. ×52.
(B) Photomicrograph of invasive adenocarcinoma in the glandular stomach of a rat in Group 1. ×52.
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chemical examination to check cell proliferation of the non-neoplastic tissues of the stomach by using PCNA antibody. Briefly, endogenous peroxidase activity was blocked by immersing the sections in methanol with 3% hydrogen peroxidase for 10 min, and then the sections were rinsed 3 times with Tris-HCl (pH 7.0). They were incubated in 1% bovine serum albumin for 40 min and then incubated with PCNA antibody (Oncogene Research Products, Cambridge, MA) at a 1:100 dilution in Tris-HCl for 90 min at room temperature. They were rinsed with Tris-HCl again, and PCNA staining was done by the labeled streptavidin biotin (LSAB) method using an LSAB 2 kit (DAKO Co., Carpinteria, CA). The peroxidase binding sites were detected by staining with 3,3'-diaminobenzidine. Counterstaining was performed using Mayer's hematoxylin. We counted the number between the PCNA-positive cells in the uppermost layer and the bottom of the non-lesional gastric mucosal columns of the pylorus. PCNA-labeling index was determined by measuring the number of PCNA-positive nuclei as a proportion of the total nuclei, 20 columns per slide, 5 slides per group.

**Statistical analysis**

The incidence of adenocarcinoma and adenomatous hyperplasia, animal body weights, and PCNA-labeling indices were compared among each group. Fisher's exact test (Fisher, 1950), Welch's method and Student's t-test (Gad and Weil, 1982) were used to determine the significance of differences. Differences were considered significant at the p<0.05 level.

![Graph showing changes in body weight of each group.](image)

**Fig. 2** Changes in body weight of each group.

* Significantly different from Group 5 by Welch's method (p<0.001).
RESULTS

The mean body weights of MNU-treated groups, (Groups 1 through 3), were significantly smaller than those of Group 5 (p<0.001) (Fig. 2).

Histopathological findings are summarized in Table 1. In this study, no forestomach lesions were recognized in any rats of 5 Group. Furthermore, no clear evidence for the toxicity of CA was confirmed in any organs of rats in the group exposed to CA alone. Most tumors of the glandular stomach were found in the lesser curvature of the pyloric region. Many of them presented as ulcerative nodules with elevated borders, their diameters were 4 mm to 12 mm, and they were histologically well or moderately differentiated adenocarcinomas. The incidence of the carcinoma was 60.0% (12 of 20 rats) in Group 1, 33.3% (7 of 21 rats) in Group 2 and 23.5% (4 of 17 rats) in Group 3. The incidence of carcinoma in Group 3 was significantly lower than that of Group 1 (p<0.03). The incidence of carcinoma in Group 2 was rather lower than in Group 1, but the difference was not significant (p=0.081). The incidence of adenomatous hyperplasia was 80.0% (16 of 20 rats) in Group 1, 28.6% (6 of 21 rats) in Group 2 and 58.8% (10 of 17 rats) in Group 3. The incidence of hyperplasia in Group 2 was also significantly lower than in Group 1 (p<0.002).

The scores of the PCNA-labeling index of the epithelial cells in the stomach are illustrated in Table 2. The index of Groups 2 (10.4±0.7) and 3 (9.9±0.3) was significantly smaller than that of Group 1 (15.1±3.7) (p<0.001).

DISCUSSION

In the present study, incidences of stomach carcinomas and adenomatous hyperplasia in the groups given MNU and CA (Group 2 or 3) were lower than that of the group given carcinogen alone (Group 1). The results suggest that exposure to CA during the post-initiation phase suppresses MNU-induced glandular stomach carcinogenesis in rats. Certainly, no clear dose response was confirmed for the effects of CA on the incidence of carcinomas. However, it is assumed that optimal doses will be importantly concerned with expression of inhibitory effects of chemopreventive agents. Actually, we have experienced several such cases (Tanaka et al., 1989).

CA and other phenolic acids are known to exert antimutagenic effects and/or anticarcinogenic effects.

Table 1. Incidence of gastric carcinoma and adenomatous hyperplasia in each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>adenocarcinoma (%)</th>
<th>adenomatous hyperplasia(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MNU alone</td>
<td>20</td>
<td>12 (60.0)</td>
<td>16 (80.0)</td>
</tr>
<tr>
<td>2</td>
<td>MNU→500 ppm CA</td>
<td>21</td>
<td>7 (33.3)</td>
<td>6 (28.6) b)</td>
</tr>
<tr>
<td>3</td>
<td>MNU→250 ppm CA</td>
<td>17</td>
<td>4 (23.5) a)</td>
<td>10 (58.8)</td>
</tr>
<tr>
<td>4</td>
<td>500 ppm CA alone</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Non-treatment</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a) Significantly different from Group 1 by Fisher’s exact test (p<0.03)
b) Significantly different from Group 1 by Fisher’s exact test (p<0.002)

Table 2. PCNA-labeling index in each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of columns</th>
<th>PCNA-labeling index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MNU alone</td>
<td>100</td>
<td>15.1±3.7 a b)</td>
</tr>
<tr>
<td>2</td>
<td>MNU→500 ppm CA</td>
<td>100</td>
<td>10.4±0.7 c)</td>
</tr>
<tr>
<td>3</td>
<td>MNU→250 ppm CA</td>
<td>100</td>
<td>9.9±0.3 c)</td>
</tr>
<tr>
<td>4</td>
<td>500 ppm CA alone</td>
<td>100</td>
<td>9.4±1.7</td>
</tr>
<tr>
<td>5</td>
<td>Non-treatment</td>
<td>100</td>
<td>9.0±0.6</td>
</tr>
</tbody>
</table>

a) Mean±S.D.
b) Significantly different from Group 5 by Welch’s method (p<0.001).
c) Significantly different from Group 1 by Welch’s method (p<0.001).
(Kuenzig et al., 1984; Mori et al., 1986; Tanaka et al., 1990). For these effects of the phenolic compounds, complicated mechanisms including modification of metabolic systems, antioxidative activity, prevention or destruction of free radicals and reducing ornithine decarboxylase levels are suggested to be underlying factors (Frenkel et al., 1993; Iwahashi et al., 1990; Gali et al., 1991).

Previously, a related phenolic, caffeic acid, was reported to enhance forestomach carcinogenesis after the initiation of diethylnitrosamine, MNU, N-butyl-N-(4-hydroxybutyl)nitrosamine, 2,2′-dihydroxy-di-n-propylaminoamine and 1,2-dimethylhydrazine (Hirose et al., 1997). In this study, no neoplastic changes were recognized in the forestomach. It is suggested that caffeic acid and CA, which is an ester of caffeic acid, have different modes of action in the rodent stomach.

Cell proliferation is known to have important roles for multistage carcinogenesis in different organs (Cayama et al., 1978). In this study, the PCNA labeling index of the stomach mucosa in the groups given CA and MNU was smaller than that of the group given MNU alone. Such results also suggest that control of cell proliferation by CA is related to chemopreventive activity, as indicated in our earlier work (Mori et al., 1995; Mori et al., 1997; Tanaka et al., 1993).

In conclusion, this study indicates that CA, a plant phenolic acid, may be a promising agent for prevention of human stomach cancers.

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