A Novel Prodrug of 4′-Geranyloxy-Ferulic Acid Suppresses Colitis-Related Colon Carcinogenesis in Mice

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The inhibitory effects of a novel prodrug, 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoyl-L-alanyl-L-proline (GAP), of the secondary metabolite 4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic acid (4′-geranyloxy-ferulic acid), on colon carcinogenesis was investigated using an azoxymethane (AOM)/dextran sodium sulfate (DSS) model. GAP was synthetically derived from ferulic acid. Male CD-1 (ICR) mice initiated with a single intraperitoneal injection of azoxymethane (10 mg/kg body weight) were promoted by 1% (wt/vol) DSS in drinking water for 7 days. They were then given modified AIN-76A diet containing 0.01% or 0.05% GAP for 17 wk. At Week 20, the development of colonic adenocarcinoma was significantly inhibited by GAP feeding at dose levels of 0.01% (60% incidence (P = 0.0158) with a multiplicity of and 1.13 ± 1.13 (P < 0.05]) and 0.05% [53% incidence (P = 0.0057) with a multiplicity of 0.08 ± 1.08 (P < 0.01)], when compared to the AOM/DSS group (95% incidence with a multiplicity of 3.10 ± 3.06). Dietary GAP modulated the mitotic and apoptotic indexes in the crypt cells and lowered 8-hydroxy-2′-deoxyguanosine (8-OHdG)-positive cells in the colonic mucosa. Urinary level of 8-OHdG was lowered by GAP feeding. Additionally, dietary GAP elevated the immunoreactivity of an inducible form of heme oxygenase 1 in the colonic mucosa. Our results indicate that GAP is able to inhibit colitis-related colon carcinogenesis by modulating proliferation and oxidative stress in mice.

INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer deaths in the Western countries. Globally, the mortality of CRC was 655,000 deaths per year in 2005 (1). Inflammation was known to be linked with cancer development in several tissues (2). CRC is one of the most serious complications of inflammatory bowel disease (IBD), including ulcerative colitis and Crohn’s disease. The risk of CRC increases with increasing extent and duration of the disease (3). For treatment or chemoprevention of IBD and IBD-related CRC, many drugs and chemopreventive agents were introduced (4). A large amount of the drugs are absorbed from the upper gastrointestinal tract, stomach, and small intestine and cause certain side effects. Therefore, it is preferable to deliver the drug site specifically to the colon.

Several synthetic or natural compounds exerting antioxidative and/or anti-inflammatory properties have been proposed as cancer chemopreventive agents (5–7). We previously reported that ferulic acid (R = H, Fig. 1a), abundant in edible plants, such as rice and black raspberries, is able to inhibit chemically induced carcinogenesis in rodents (8). Other investigators have reported data supporting our findings (9,10). A secondary metabolite biosynthetically derived from ferulic acid, 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic acid (R = geranyl, Fig. 1a), is supposed to exert cancer chemopreventive effect (11).

Recently, novel natural and semisynthetic compounds with anti-inflammatory activity (12) have been reported to be effective chemopreventive agents against carcinogenesis in preclinical animal studies, such as collinin (7-geranyloxy-8-methoxy-coumarin) (13), auraptene (13,14) and the ethyl ester...
of 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic acid (EGMP) (15,16). Because inflammation is a universal and physiological response in the process of carcinogenesis (2,17–19), the in vivo and in vitro anti-inflammatory properties of these compounds have been demonstrated (20,21). Auraptene and collin were reported to cause complete inhibition of platelet aggregation induced by arachidonic acid and platelet-activating factor in vitro (22), to act as good chemopreventers in colitis-related mouse colon tumorigenesis (13). In addition, our synthetic derivative, EGMP, has shown various interesting biological effects such as suppression of inducible nitric oxide (iNOS) and cyclooxygenase (COX)-2 protein expression in RAW 264.7 cells induced by lipopolysaccharide and interferon gamma (23) and colon and tongue cancer chemoprevention by dietary feeding in rats (15,16). Furthermore, some myo-inositol esters of 4′-geranyloxy-ferulic acid have good inhibitory effects on phorbol ester-induced superoxide generation and Epstein-Barr virus activation (24). All these esters could be hydrolyzed to the parent acid once inside the cells. So the true active compound exerting the above-cited observed biological effects would be 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic acid. Then, it could become a novel candidate as chemopreventive agent of various cancer types and as an anti-inflammatory compound. Pharmacological and chemical properties of the latter acid were recently reviewed (11). To achieve a novel approach in the prevention of CRC by drugs administered in diet, we carried out the synthesis of a novel prodrug, 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoyl-L-alanyl-L-proline (GAP, molecular weight = 498.62). This novel prodrug of 4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic acid would be delivered in high concentration in the large bowel (25). Furthermore, its mechanism of activation would ensure chemical and enzymatic stability while passing through the stomach and small intestine by in vitro study (25).

For investigation of the pathogenesis (26–28) and chemoprevention (13,29) of inflammation-related CRC, our mouse model of inflammation related 2-stage colon carcinogenesis with a colonic carcinogen, and a colitis-inducing agent, dextran sodium sulfate (DSS) (15), is useful (30,31). In this model, the powerful tumor promoting effect of DSS is closely related to oxidative/nitrosative stress caused by DSS-induced colitis (26–28). This suggests that oxidative/nitrosative DNA damage by inflammation is involved in carcinogenesis, and thus it is important to control the events leading to inflammation-related carcinogenesis (17). In humans, oxidative stress also plays a key role in the pathogenesis of IBD-related intestinal damage (32). 8-Hydroxy-2′-deoxyguanosine (8-OHdG) production is induced by the oxidation of deoxyguanosine (dG), which is one of the components of DNA. Hydroxyl radicals (·OH) directly act on dG to form 8-OHdG. It is stable in humans and is excised by repair enzymes like 8-oxoguanine DNA glycosylase 1 and excreted in urine. 8-OHdG formation in DNA may also be related to tumorigenesis because many mutagens, tumor promoters, and carcinogens are known to generate oxygen radicals, and this generation of oxygen radicals in vivo is thought to be relevant to carcinogenesis (33). Elevation of urinary and tissue 8-OHdG levels are also known in IBD patients (32).

In the current study, we investigated whether dietary GAP exerts cancer chemopreventive ability in colitis-associated colon carcinogenesis using our mouse model (34). Also, effects of GAP on oxidative stress induced by azoxymethane (AOM) and/or DSS were evaluated by measuring urinary level of 8-OHdG and immunohistochemical expression of 8-OHdG in the colonic mucosa. Additionally, we measured immunohistochemical expression of an important antioxidant enzyme, heme oxygenase (HO)-1, that is involved in the heme degradation process in the colonic mucosa because the significance of targeted induction of HO-1 as a strategy to achieve chemoprevention and chemoprotection is suggested (35).

MATERIAL AND METHODS

Animals, Chemicals, and Diets

Male Crj: CD-1 (ICR) mice (Charles River Japan, Tokyo, Japan), aged 5 wk, were used in this study. The animals were maintained in Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (5 mice/cage) with free access to tap water and a pelleted basal Charles River Formula-1 diet (Oriental Yeast Co., Ltd., Tokyo, Japan) during quarantine under controlled conditions of humidity (50 ± 10%), lighting (12-h light/dark cycle), and temperature (23°C ± 2°C). They were quarantined for 7 days after arrival and randomized by body

FIG. 1. Chemical structure of (a) ferulic acid, R = H and 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic acid, R = geranyl, and (b) 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoyl-L-alanyl-L-proline (GAP, molecular weight = 498.62).
weight into experimental and control groups. A colonic carcino-
gen AOM was purchased from Sigma-Aldrich Chemical Co. (St.
Louis, MO). DSS with a molecular weight of 36,000 to 50,000
was purchased from ICN Biochemicals (Aurora, OH). DSS for
induction of colitis was dissolved in water at 1% (wt/vol). GAP
was synthesized, as described previously (25). Experimental di-
ets containing 0, 0.01, and 0.05% GAP in modified AIN-76A
(36) were prepared weekly in our laboratory and stored in a
cold room. Animals had access to food and water at all times.
Food cups were replenished with fresh diet everyday. All han-
dling and procedures were carried out in accordance with the
Institutional Animal Care Guidelines.

Experimental Procedures

The Institutional Animal Care and Use Committee evaluated
all animal procedures associated with the present study and
assured that all proposed methods were appropriate.

A total of 60 male ICR mice were divided into 5 experimental
and control groups. Mice in Groups 1 through 3 were initiated
with AOM by single intraperitoneal injection (10 mg/kg body
weight). Starting 1 wk after the injection, 1% DSS in drinking
water was administered to mice for 7 days and then followed
without any further treatment for 18 wk. Mice of Group 1 were
maintained on modified AIN-76A diet throughout the study. Mice
of Groups 2 and 3 were fed modified AIN-76A diets containing
0.01% GAP (Group 2) and 0.05% GAP (Group 3) for
17 wk, respectively, starting 1 wk after the cessation of DSS
exposure. Group 4 did not receive AOM and DSS and were fed
AIN-76A diet containing 0.05% GAP. Group 5 was fed modified
AIN-76A diet and served as an untreated control. At the end of
study (Week 20), all mice were sacrificed by CO2 asphyxiation.
They underwent careful necropsy, with emphasis on the colon,
 liver, kidney, lung, and heart.

At necropsy, the colons were flushed with saline, excised,
their length measured (from ileocecal junction to the anal verge),
cut open longitudinally along the main axis, and then washed
with saline. They were cut and fixed in 10% buffered formalin
for at least 24 h. Histological examination was performed on
paraffin-embedded sections after hematoxylin and eosin (H & E)
staining. For scoring, large intestinal inflammation was
graded according to the following morphological criteria (38):
Grade 0, normal appearance; Grade 1, shortening and loss of
the basal 1/3 of the actual crypts with mild inflammation in the
mucosa; Grade 2, loss of the basal 2/3 of the crypts with mod-
erate inflammation in the mucosa; Grade 3, loss of the entire
crypts with severe inflammation in the mucosa and submucosa
but with retention of the surface epithelium; and Grade 4, pres-
ence of mucosal ulcer with severe inflammation (infiltration of
neutrophils, lymphocytes, and plasma cells) in the mucosa, sub-
mucosa, muscularis propria, and/or subserosa. The scoring was
made on the entire colon with or without proliferative lesions
and expressed as a mean average score/mouse.

Counting Mitotic and Apoptotic Cells and Crypt Heights

To identify intramucosal apoptotic and mitotic cells in the
crypts, paraffin-embedded sections from the distal colon were
stained with H & E and evaluated under a light microscope for
apoptotic and mitotic cells at a magnification of 400. Apoptotic
cells were identified by cell shrinkage, homogeneous basophilic
and condensed nuclei, nuclear fragments (apoptotic bodies),
marked eosinophilic condensation of the cytoplasm, and sharply
delineated cell borders surrounded with a clear halo (39). The
apoptotic and mitotic indexes in the colonic crypts were de-
termined on longitudinal sections that allowed evaluation of
the whole crypt from the top to the base. One colonic section
(from the distal part) per mouse was studied and scored. Ran-
domly chosen crypts (28–56 crypts/colon) with well-oriented
crypt structure from the mouth to the base were evaluated for
counting apoptosis and mitosis. The apoptotic index (AI) and
mitotic index (MI) nuclei were determined by dividing the total
number of apoptotic or mitotic cells by the number of epithelial
cells evaluated.

Immunohistochemistry of 8-OHdG and HO-1

Immunohistochemistry for 8-OHdG and HO-1 was performed
on 4 µm-thick paraffin-embedded sections from the
colons of mice in each group. The paraffin-embedded sections
were heated for 30 min at 65°C, deparaffinized in xylene, and
rehydrated through graded ethanol at room temperature. A 0.05
M Tris hydrochloride buffer (pH 7.6) was used to prepare so-
lutions and for washes between various steps. Incubations were
performed in a humidified chamber. Sections were treated for
40 min at room temperature, with 2% bovine serum albumin,
and incubated overnight at 4°C with primary antibodies such
as anti-8-OHdG mouse monoclonal antibody (diluted 1:100;
Institute of Aging, Japan) and anti-HO-1 rabbit polyclonal anti-
body (diluted 1:200, SPA-896; StressGen Biotechnologies, Ann
Arbor, MI). To reduce the nonspecific staining of mouse tissue
by the mouse antibodies, a Mouse On Mouse immunoglobulin G
blocking reagent (Vector Laboratories, Inc., Burlingame, CA)
was applied. For 8-OHdG and HO-1 immunohistochemistry,
normal rabbit serum was used to block background staining.
Staining was performed using a DAKO En Vision kit (DAKO,
Glostrup, Denmark) or Vectastain Elite ABC Kit (Vector Labo-
ratories). At the last step, the sections were counterstained with
hematoxylin. As a negative control, omission of the primary anti-
body was used. Two observers (T. Tanaka and S. Sugie) were

Scoring of Inflammation in the Large Bowel

Inflammation in the large bowel was scored on the H & E-
stained sections. For scoring, large intestinal inflammation was
graded according to the following morphological criteria (38):
Grade 0, normal appearance; Grade 1, shortening and loss of
the basal 1/3 of the actual crypts with mild inflammation in the
mucosa; Grade 2, loss of the basal 2/3 of the crypts with mod-

unaware of the treatment group to which the slide belonged and evaluated the immunoreactivity with grading between 0 and 5: 0 (<15% of the colonic mucosa examined shows positive reactivity), 1 (16–30% of the colonic mucosa examined shows positive reactivity), 2 (31–45% of the colonic mucosa examined shows positive reactivity), 3 (46–60% of the colonic mucosa examined shows positive reactivity), 4 (61–75% of the colonic mucosa examined shows positive reactivity), and 5 (>75% of the colonic mucosa examined shows positive reactivity).

**Urinary 8-OHdG Analysis**

To determine in vivo oxidative stress, urinary level of 8-OHdG was measured. One day before the sacrifice, 5 animals were selected randomly from each treatment group and placed individually into metabolic cages for urine collection. Urine was collected from each animal over a period of 3 h and frozen at −80°C until analysis. Urinary level of 8-OHdG was determined by competitive enzyme-linked immunoabsorbent assay (Genox, Baltimore, MD) and corrected for urinary creatinine concentrations.

**Statistical Evaluation**

Where applicable, data were analyzed using 1-way analysis of variance with Tukey–Kramer multiple comparisons test (GraphPad Instat version 3.05, GraphPad Software, San Diego, CA) with $P < 0.05$ as the criterion of significance. The Fisher’s exact probability test was used for comparison of the incidence of lesions between 2 groups.

**RESULTS**

**General Observation**

During the experiment, some animals that received AOM/DSS (Group 1) or AOM/DSS→GAP (Groups 2 and 3) had bloody stool, but the symptom disappeared soon after stopping of DSS treatment. At Weeks 18 to 20, some mice of these groups had bloody stool again and anal prolapse with rectal tumor. There was no significant change between the experimental groups with regards to the parameters tested (body weight, liver weight, relative liver weight, spleen weight, kidney weight, and colon length). Further, no significant pathological alternations were found in these organs except the colon.

**Pathological Findings**

Macroscopically, nodular and polypoid colonic tumors were observed in the middle and distal colon of mice in Groups 1 through 3. These tumors were histopathologically tubule adenoma (Fig. 2A) or adenocarcinoma (well-/moderately differentiated; Fig. 2B). Some adenocarcinomas invaded into submucosa or serosa. Dysplastic crypts (Fig. 2C) were also observed surrounding neoplasms. Enlarged lymph nodes with inflammation were present around the large bowel with tumors. Mice of Groups 4 (GAP alone) and 5 (untreated) had no tumors in all the organs examined including the colon.

**FIG. 2.** Representative colonic lesions induced by azoxymethane/dextran sodium sulfate in mice (Group 1): A: a tubular adenoma, B: a tubular adenocarcinoma with moderately differentiated, and C: dysplastic crypts (circled). Photos inserted in Fig. 2A and 2B are low power of views for each lesion (original magnifications are ×2 in 2A and ×4 in 2B). Figure represents hematoxylin and eosin stain, and bars inserted indicate magnification (µm).
TABLE 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Incidence (No. of Mice With Tumors)</th>
<th>Multiplicity (No. of Tumors/Mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>AD</td>
</tr>
<tr>
<td>1</td>
<td>AOM/DSS (20)</td>
<td>20 (100%)</td>
<td>19 (95%)</td>
</tr>
<tr>
<td>2</td>
<td>AOM/DSS → 0.01% GAP (15)</td>
<td>10 (67%)</td>
<td>8 (53%)</td>
</tr>
<tr>
<td>3</td>
<td>AOM/DSS → 0.05% GAP (15)</td>
<td>10 (67%)</td>
<td>8 (53%)</td>
</tr>
<tr>
<td>4</td>
<td>0.05% GAP (5)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>5</td>
<td>None (5)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

*aAbbreviations are as follows: AD, adenoma; ADC, adenocarcinoma; AOM, azoxymethane; DSS, dextran sodium sulfate; GAP, 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoyl-L-alanyl-L-proline.

*bMean ± SD.

Significantly different from the AOM/DSS group by Fisher’s exact probability test, P = 0.0002.

Significantly different from the AOM/DSS group by Fisher’s exact probability test, P = 0.0057.

Significantly different from the AOM/DSS group by Fisher’s exact probability test, P = 0.0158.

Significantly different from the AOM/DSS group 1-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test, P < 0.05.

Significantly different from the AOM/DSS group 1-way ANOVA with Tukey–Kramer multiple comparisons test, P < 0.01.

The incidences and multiplicities of colon tumors are listed in Table 1. Group 1 (AOM + DSS) had 95% incidence of colon adenocarcinoma with a multiplicity of 3.10 ± 3.06. The incidences of colon adenocarcinoma of Groups 2 (AOM/DSS → 0.01% GAP, 60%) and 3 (AOM/DSS → 0.05% GAP, 53%) were significantly smaller than that of Group 1 (P = 0.0158 and P = 0.0057, respectively). Also, the multiplicities of colon adenocarcinoma of Groups 2 (1.13 ± 1.13, P < 0.05) and 3 (0.80 ± 1.08, P < 0.01) were significantly smaller than that of Group 1.

Inflammation Score in the Colon

Fig. 3A illustrates data on colonic inflammation scores at Week 20. The inflammation score of Group 1 (2.45 ± 0.89) was the greatest. The scores of Groups 2 (1.67 ± 0.82, P < 0.05) and 3 (1.07 ± 0.80, P < 0.001) were significantly lower than that of Group 1. Colonic inflammation in the mice of Groups 4 and 5 was slight, if present.

Indices of Mitosis and Apoptosis in Colonic Crypts

The data on the epithelial proliferative kinetics in the “normal appearing” distal colon are illustrated in Figs. 3B through 3D. As shown in Fig. 3B, the mean number of crypt cell MI of Groups 1 was significantly higher (4.33 ± 2.16, 2.37-fold increase; P < 0.001) than that of Group 5 (1.83 ± 1.60). The dietary administration of GAP (Groups 2 and 3) reduced the mean MI in a dose-dependent manner when compared to Group 1 (4.33 ± 2.16): 27% reduction by 0.01% GAP (Group 2, 3.17 ± 1.17, P < 0.01) and 54% reduction by 0.05% GAP (Group 3, 2.00 ± 0.89, P < 0.001). Feeding with 0.05% GAP alone (Group 4, 1.83 ± 1.17) did not affect the MI in the crypts when compared to an untreated control (Group 5, 1.83 ± 1.60). As indicated in Fig. 3C, the mean AI of group 1 (1.80 ± 0.84, P < 0.05) was significantly greater than that of Group 5 (1.20 ± 0.84). The values of Groups 2 (2.20 ± 0.84) and 3 (3.00 ± 0.71) were larger than that of Group 1, and the increase of Group 3 was statistically significant (P < 0.001). The mean AI of Groups 4 (1.40 ± 0.55) and 5 were comparable. As for the crypt column height (number of cells/crypt, Fig. 3D), the value in Group 1 (44.2 ± 4.97, P < 0.001), being the lowest among the groups, was significantly smaller than Group 5 (61.8 ± 8.76). The crypt column heights of Groups 2 (45.8 ± 6.06) and 3 (57.4 ± 12.6) were larger than Group 1, and the increase of Group 3 was statistically significant (P < 0.001). The value of Groups 4 (58.2 ± 5.81) and 5 were comparable.

Scores of 8-OHdG and HO-1 Immunohistochemistry

Mean scores of HO-1 and 8-OHdG immunohistochemistry are illustrated in Figs. 4A and 4B, respectively. The mean score of HO-1 immunohistochemical positivity of Group 1 (2.10 ± 0.88) was significantly greater than that of Group 5 (0.60 ± 0.89, P < 0.05; Fig. 4A). The score of Group 3 (3.40 ± 1.07) was significantly larger than Group 1. The value of Group 2 (3.00 ± 0.82) was greater than that of Group 1, but the increase was insignificant. As shown in Fig. 4B, the mean score of 8-OHdG immunohistochemical positivity of group 1 (3.90 ± 0.88) was significantly greater than that of Group 5 (0.40 ± 0.55, P < 0.001; Fig. 4B). The scores of Groups 2 (2.40 ± 0.52, P < 0.001) and 3 (1.80 ± 0.79, P < 0.001) were significantly lower than Group 1.
FIG. 3. A: Inflammation score (mean ± SD) of colon from each group. Photo shows inflammation score, Grade 2, in the colon of a mouse from Group 1; hematoxylin and eosin (H & E) stain; a bar inserted indicates magnification (µm). B: Mitotic index (MI, mean ± SD) of crypts of each group. For Fig. 3B photos, arrowheads in green are mitoses, and those in red are apoptotic nuclei or apoptotic bodies; H & E stain; a bar inserted indicates magnification (µm). C: Apoptotic index (AI, mean ± SD) of crypts of each group. For Fig. 3C photo, an arrowhead in green is a mitotic nucleus, and those in red are apoptotic nuclei or apoptotic bodies; H & E stain; a bar inserted indicates magnification (µm). D: Crypt height (number of cells per crypt, mean ± SD) of each group. For the Fig. 3D photo, arrowheads in green are mitoses, and an arrowhead in red is an apoptotic nucleus or apoptotic body; H & E stain; a bar inserted indicates magnification (µm). AOM, azoxymethane; DSS, dextran sodium sulfate; GAP, 3-(4’-geranyloxy-3’-methoxyphenyl)-2-trans-propenoic acid-

Urinary Level of 8-OHdG

Data on urinary 8-OHdG (ng/mg creatinine) are shown in Fig. 4C. The level of Group 1 (7.10 ± 1.60, P < 0.001) was significantly greater than that of Group 5 (3.20 ± 1.79). The values of Groups 2 (4.30 ± 1.57, P < 0.01) and 3 (3.70 ± 1.334, P < 0.001) were significantly smaller than that of Group 1. The levels of Groups 4 (4.00 ± 1.22) and 5 were comparable.

DISCUSSION

The results of this study clearly indicate that a novel prodrug of the already known colon cancer chemopreventive agent 3-(4’-geranyloxy-3’-methoxyphenyl)-2-trans-propenoic acid effectively inhibited AOM/DSS-induced, colitis-related, colonic carcinogenesis without any adverse effects in mice. Dietary feeding with GAP exerted its cancer chemopreventive ability by modulating cell proliferation, suppressing oxidative damage (tissue expression and urinary level of 8-OHdG), and enhancing an antioxidant enzyme, HO-1, in the inflamed colon. This is the first report showing that a prodrug, GAP, exerts cancer chemopreventive ability in colitis-related colon carcinogenesis.

The incidence and multiplicity of colonic tumors in the mice received AOM and 1% DSS in the current study were higher
A PRODRUG OF FERULIC ACID INHIBITS COLITIS-RELATED CARCINOGENESIS

FIG. 4. A: Score (mean ± SD) of heme oxygenase (HO)-1 immunoreactivity. Photo shows strong HO-1 immunoreactivity (Grade 2) of colonic mucosa (same as an inset in Fig. 4) from a mouse of Group 1. Strong positive reaction is present in cryptal cells and inflammatory cells infiltrated into the inflamed colon; HO-1 immunohistochemistry; a bar inserted indicates magnification (µm). B: Score (mean ± SD) of 8-hydroxy-2′-deoxyguanosine (8-OHdG) immunoreactivity. Photo shows strong 8-OHdG immunoreactivity (Grade 3) of colonic mucosa from a mouse of Group 1. Strong positive reaction is present in inflammatory cells in the inflamed colon, and weak reaction is seen in the surface of crypt cells; 8-OHdG immunohistochemistry; a bar inserted indicates magnification (µm). C: Urinary 8-OHdG level (ng/mg creatinine, mean ± SD) of each group. The measurement was done by competitive enzyme-linked immunoabsorbent assay and corrected for urinary creatinine concentration. AOM, azoxymethane; DSS, dextran sodium sulfate; GAP, 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoyl-L-alanyl-L-proline.

than our previous dose-response study (40); this may be due to the difference of intake of 1% DSS-containing drinking water: 11.06 ± 0.05 ml/mouse/day in this study and 8.60 ± 0.94 ml/mouse/day in a previous investigation (40). Dietary GAP was able to modulate the endpoints measures in a dose-dependent manner, but the effects on tumor (total tumors and adenoma) multiplicity were comparable. The reason for this is unknown. However, the effects of GAP on the multiplicity of colonic adenocarcinoma suggest the inhibition of progression and the presence of dose-dependent efficacy. Therefore, we should determine the dose-dependency of the inhibition by GAP utilizing 3 or more doses in future studies.

Like ferulic acid (41), our data on 8-OHdG in the colon and urine suggests the antioxidative potential for GAP. Dietary administration of GAP effectively lowered the tissue expression of 8-OHdG in the inflamed colon as well as the urinary level of 8-OHdG. One of the markers of oxidative stress is 8-OHdG, which results from free radical damage to guanine (42). Elevated levels of 8-OHdG have been correlated with malignancy in the colon of rats (43) and humans (44). 8-Oxodeoxyguanosine, the tautomer of 8-OHdG, induces errors in DNA replication, specifically G-to-T transversions (45). Phenolic antioxidants in foods have been shown to reduce markers of oxidative stress and suppress carcino genesis in certain tissues (46). For example, catechins in tea reduce urinary 8-OHdG content and are effective chemopreventives in the F344 model of colon carcinogenesis (47). In IBD patients, oxidative DNA damage and decrease in antioxidant activity are known (32). We previously reported increased oxidative damage in the inflamed colon of mice treated with DSS (26–28), and modulation of oxidative damage could prevent cancer occurrence (13,29). As found in a phase IIa clinical chemoprevention trial with green tea polyphenols in which urinary 8-OHdG can be monitored to determine oxidative stress condition (48), urinary concentration of 8-OHdG serves as a practical biomarker of oxidative DNA damage in preclinical animal studies.

In the current study, the treatment with GAP in diet significantly lowered colonic inflammation induced by DSS. Because chronic inflammation involves in carcinogenesis, suppression of chronic inflammation through modulation of expression of several pro-inflammatory gene products that mediate a critical role in several events of carcinogenesis may result in cancer chemoprevention (49). Ferulic acid and EGMP have anti-inflammatory effects and inhibition of iNOS expression and thereby suppress carcinogenesis (8,15,23). In fact, our recent study demonstrated that modulation of inflammation and expression of COX-2 and iNOS in the colon contributes to suppression of colitis-related colon carcinogenesis (50). Because several molecular targets for suppression of inflammation-associated carcinogenesis were proposed (51), further studies are warranted for detailed mechanisms by which GAP inhibits inflammation-related carcinogenesis.

Interesting findings observed in this study are that GAP treatment enhanced HO-1 expression in the colon of mice that
received AOM/DSS. HO-1 participates in endogenous cellular defense against oxidative stress (52). HO-1 confers cytoprotection against injury in a variety of organs and tissues where inflammatory processes are implicated. HO plays a central role in heme metabolism (52). At the same time, it protects cells from injury evoked by various oxidative stresses. HO-1 expression is carefully controlled in vivo with regard to its location and the magnitude. Furthermore, it was recently shown that HO-1 is involved in the immune regulation (53). These findings suggest HO-1 protein in vivo as a novel therapeutic intervention to control various forms of inflammatory disorders. Additionally, HO-1 is reported to inhibit inflammation through mitogen-activated protein kinase (MAPK) activation by induction of CO (54). The strategy that cell injury caused by oxidative stress and subsequent inflammatory condition are reduced and treated by induction of HO-1 expression is sound. However, because HO-1 is an inducible enzyme, we should control the expression quantitatively and with time. Several dietary constituents can modulate HO-1 expression (55). In addition to in vitro studies using known chemopreventive agents (56), there is an in vivo cancer chemoprevention study in which sulforaphane inhibits rat mammary carcinogenesis by induction of HO-1 (57). Upregulation of HO-1 by quercetin protects human hepatocytes from ethanol-induced oxidative stress via the MAPK/Nrf2 pathways (58). Also, Nrf2-deficient mice are susceptible to DSS-induced colitis (59). Therefore, safer natural compounds and their prodrug, such as GAP, may be used for prevention of inflammation-related cancer development.

When compared to the inhibitory effects of different chemicals on the multiplicity of colonic adenocarcinoma using this animal model, inhibitory potency was in the following order: ursoodeoxycholic acid (29) > nimesulide (50) > colilcon (13) > auraptene (13) > GAP (this study) > sulfasalazine (29) > pitavastatin (38) > troglitazone (50) > bezafibrate (50). However, we should consider the findings were observed in different experimental conditions such as dose of DSS and test chemicals and duration of exposure of chemicals. In addition, we need future experiments to check the effect of GAP on the kinetics of colon cancer development in our model system.

In conclusion, a novel prodrug of 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoic acid, resulting from the conjugation of this acid to the dipeptide L-Ala-L-Pro, is effective in inhibiting colon cancer development in a 2-stage, colitis-related, mouse colon carcinogenesis through modulation of inflammation, oxidative stress, and cell proliferation in the inflamed colon of mice that received AOM and DSS. Our findings support the development of novel, site-specifically delivered prodrugs for colon cancer prevention in the inflamed colon.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Cancer Research, for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare of Japan; a Grant-in-Aid (No. 18592076 to T. Tanaka, 17015016 to T. Tanaka, and 18880030 to Y. Yasui) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and a grant (H2007–12 to T. Tanaka and S2006–9 to Y. Yasui) for the Project Research from the High-Technology Center of Kanazawa Medical University. We also thank Italian Ministero dell’Istruzione, Università e Ricerca (MIUR) for financial support for the synthesis of the title prodrug.

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