to inhibiting proliferation and inducing apoptosis in cancer cells (14, 15). For instance, EGCG inhibits cell growth, induces apoptosis, and inhibits the expression of cyclooxygenase (COX)-2 by inhibiting the activation of epidermal growth factor receptor family of receptor tyrosine kinases (RTK) and their downstream signaling molecules, such as extracellular signal-regulated kinase and Akt proteins, in human colorectal cancer cells (20, 21). In addition, EGCG also inhibits the activation of IGF-IR, which belongs to a separate family of RTKs, in human colorectal cancer and liver cancer cells (22, 23).

The development of azoxymethane-induced aberrant crypt foci (ACF) and β-catenin accumulated crypts (BCAC), both of which are putative precursor lesions for colonic adenocarcinoma (24, 25), have been previously reported to be enhanced in C57BL/KsJ-*db/db* (*db/db*) mice with hyperinsulinemia and hyperleptinemia, when compared with *db/+* or +/+ mice (26). This is a useful preclinical animal model to examine the possible underlying mechanisms on how chemopreventive agents inhibit the development of obesity-related colorectal cancer because *db/db* mice are more susceptible to azoxymethane as described above, and the chemopreventive effect by specific agent is more apparent in these mice than in *db/+* or +/+ mice (27). In view of the evidence that insulin resistance plays an important role in the development of colorectal cancer via activation of the IGF/IGF-IR axis (1, 2, 11–13), and the previous studies indicating that EGCG inhibits activation of this axis in human cancer cells (22, 23), in the present study we examined in detail the effects of EGCG on the development of colonic premalignant lesions, ACF and BCAC, in *db/db* mice initiated with azoxymethane.

Materials and Methods

Animals, chemicals, and diets

Four-week-old male homozygous *db/db* mice were obtained from Japan SLC, Inc. All mice were maintained at Gifu University Life Science Research Center, according to the Institutional Animal Care Guidelines, and were housed in plastic cages with free access to drinking water (tap water with or without EGCG) and a basal diet, CRF-1 (Oriental Yeast Co., Ltd.). A colonic carcinogen azoxymethane was purchased from the Sigma Chemical Co. EGCG was obtained from Mitsui Norin Co., Ltd.

Experimental procedure

A total of 45 male *db/db* mice were divided into five groups (Fig. 1). At 5 wk of age, mice in groups 3 through 5 were s.c. injected with azoxymethane (15 mg/kg body weight) once a week for 4 wk. Group 3 was given tap water throughout the experiment. Groups 4 and 5 were given tap water containing 0.01% EGCG and 0.1% EGCG, respectively, with free access to drinking for 7 wk, starting 1 wk after the last injection of azoxymethane. Group 2 was given 0.1% EGCG alone for 8 wk. Group 1 served as an untreated control. At the termination of the study (16 wk of age), mice were sacrificed by CO_2 asphyxiation to analyze the number of colonic ACF and BCACs.

Identification of ACF and BCACs

The presence of ACF and BCACs was determined according to the standard procedures that are routinely used in our laboratory (27, 28). After the colons were fixed flat in 10% buffered formalin, the mucosal surface of the colons were stained with methylene blue and then the number of ACF were counted under a light microscope. After counting the ACF, the distal parts (5 cm from anus) of the colon were cut to count the number of BCACs. To identify intramucosal lesions BCACs, the distal part of the colon was embedded in paraffin, and then a total of 20 serial sections (4-μm thick each) per colon were made by an *en face* preparation (27, 28).

Histopathology and immunohistochemistry

Immunohistochemical analyses for β-catenin or IGF-IR were done using the labeled streptavidin-biotin method (LSAB kit; DAKO) or stain system kit (Zymed), respectively (26–28). Three serial sections were made from the paraffin-embedded tissue blocks. Two sections were subjected to H&E staining for histopathology and β-catenin immunohistochemistry to count the number of BCACs. The other section was for IGF-IR immunohistochemistry. Immunoreactivity was regarded as positive if apparent staining was detected in the cytoplasm and/or nuclei to determine the BCACs (27, 28).

Protein extraction and Western blot analysis

Total proteins were extracted from the scraped mucosa from the remaining colon of the azoxymethane-treated mice (groups 3 through 5) and equivalent amounts of proteins (40 μg/lane) were examined by a Western blot analysis (21–23, 29). The primary antibodies for IGF-IR, p-IGF-IR, p-GSK-3β, COX-2, and glyceraldehyde-3-phosphate dehydrogenase were described previously (23, 29). Anti–β-catenin antibody was obtained from Transduction Laboratories. Anti–cyclin D1 antibody was from Santa Cruz Biotechnology, Inc. An antibody to glyceraldehyde-3-phosphate dehydrogenase served as a loading control.

Clinical chemistry

At sacrifice, blood samples were collected to measure the serum concentrations of insulin, leptin, triglyceride, total cholesterol, IGF-I, and IGFBP-3. The levels of insulin, IGF-I, and IGFBP-3 were measured in the mice from groups 3, 4, and 5, and other measurements were determined in all the groups. The serum triglyceride and cholesterol were assayed as described previously (27). The serum insulin, leptin, IGF-I, and IGFBP-3 were determined by an enzyme immunoassay according to the manufacturer's protocol (R&D Systems).

Statistical analysis

The results were given as mean ± SD and were analyzed using the GraphPad Instat software program version 3.05 (GraphPad Software) for Macintosh. Differences between groups were analyzed by one-way ANOVA or, as required, by two-way ANOVA. When ANOVA showed a statistically significant effect ($P < 0.05$), comparisons of each experimental group with the control group was made using Dunnett's test, which corrects for multiple comparisons. The differences were considered significant when two-sided P was <0.05.

Results

General observations

Figure 1 shows the growth curves of all groups during this experiment. The body weight gain of the mice that received 0.1% EGCG alone (group 2) was slightly larger than that of group 1 (untreated), but the difference was not significant. The body weight gains of groups 3 (azoxymethane alone), 4 (azoxymethane plus 0.01% EGCG), and 5 (azoxymethane plus 0.1% EGCG) were smaller than those of groups 1 and 2. However, the values were comparable among the groups 3 through 5. The body weights of the mice in all groups at the end of the study are listed in Table 1. The mean body weights in the azoxymethane-treated groups (groups 3 through 5) were significantly lower than those of group 1 ($P < 0.05$). This finding is consistent with our previous report and might be associated with the toxicity of azoxymethane because db/db mice are more susceptible to azoxymethane toxicity due to lower maximum tolerated dose of this carcinogen when compared with +/+ mice (26). However, the values were comparable among

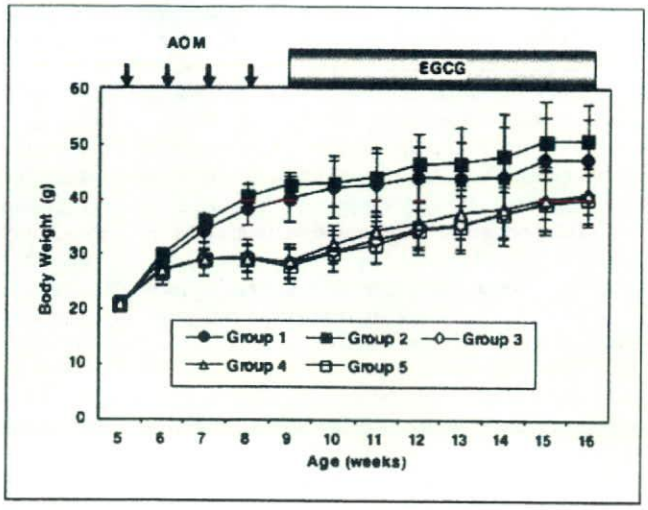

Fig. 1. Growth curves of the experimental mice. *Points*, mean; *bars*, SE. For experimental protocol in each group, see Table 1.

the groups 3 through 5 (azoxymethane alone versus azoxymethane plus EGCG–treated groups). There was no significant difference in the weights of the liver and kidney (per body weight) among these groups. A histopathologic examination also revealed no alterations, thus suggesting the absence of toxicity of EGCG in the liver and kidney of the mice in groups 2, 4, and 5 (data not shown).

Effects of EGCG on azoxymethane-induced ACF and BCAC formations in db/db mice

Table 1 summarizes the number of total ACF, large ACF with four or more crypts, and BCACs (Fig. 2A and B) in the mice of all groups. ACF and BCACs developed in the colons of all the mice that received azoxymethane (groups 3-5), but not in the colons of the mice in groups 1 and 2 that did not receive azoxymethane. In comparison with the azoxymethane alone group (group 3), treatment with a high dose (0.1%) of EGCG (group 5) significantly inhibited the development of total ACF (33% reduction, $P < 0.05$), large ACF (60% reduction, $P < 0.001$), and BCACs (57% reduction, $P < 0.01$). Similarly, administration of a low dose (0.01%) of EGCG (group 4) also reduced the numbers of total ACF (31% reduction, $P < 0.05$), large ACF (40% reduction, $P < 0.001$), and BCACs (50% reduction, $P < 0.01$).

Effects of EGCG on serum levels of triglyceride, total cholesterol, and leptin in db/db mice

The serum concentrations of triglyceride, total cholesterol, and leptin are listed in Table 2. The EGCG administration in the drinking water caused a significant decrease in the serum levels of triglyceride in groups 2 ($P < 0.01$), 4 ($P < 0.01$), and 5 ($P < 0.01$), irrespective of azoxymethane exposure. A significant decrease of triglyceride was observed in group 3 ($P < 0.01$) compared with group 1, and this finding might be explained by the body weight loss caused by azoxymethane toxicity (Table 1) because obesity is the metabolic stressor most frequently associated with hypertriglyceridemia (30). Among the azoxymethane-treated groups, the serum levels of total cholesterol also significantly decreased when the mice were given EGCG at doses of 0.01% ($P < 0.01$) and 0.1% ($P < 0.01$). The mean concentration of triglyceride and total cholesterol of group 3 were significantly lower than that of group 1 ($P < 0.01$). The serum leptin levels of groups 4 and 5 were also significantly smaller than those of group 3 ($P < 0.01$ for each comparison).

Immunohistochemical analysis of IGF-IR in BCACs

Recent studies indicate that the activation of the IGF/IGF-IR axis stabilizes and activates the Wnt/β-catenin pathway, which is involved in colorectal carcinogenesis (31, 32). Therefore, the expression of IGF-IR was examined in BCACs by immunohistochemical analysis (Fig. 2). Immunohistochemistry of IGF-IR revealed a strong reactivity in the cytoplasm and, in part, the nuclei of atypical cells in all of BCACs, when compared with their surrounding cryptal cells (Fig. 2C). This finding suggests that up-regulation of the IGF/IGF-IR axis and the accumulation of β-catenin might be associated with obesity-related colorectal carcinogenesis.

Table 1. Effects of EGCG on azoxymethane-induced ACF and BCAC formations in *db/db* mice

Group no.	Treatment	No. mice	Body weight (g)	Length of colon (cm)	Total no. of ACFs/colon	Total no. of large ACFs*/colon	Total no. of BCACs/cm^2
1	None	6	48.5 ± 9.9†	12.5 ± 0.9	0	0	0
2	0.1% EGCG	6	50.9 ± 3.8	12.0 ± 0.6	0	0	0
3	AOM alone	11	40.6 ± 5.4‡	12.3 ± 0.7	83.2 ± 7.2	33.5 ± 5.3	13.2 ± 8.5
4	AOM + 0.01% EGCG	11	41.0 ± 5.7‡	12.0 ± 0.6	57.1 ± 8.2§	20.1 ± 4.5‖	6.6 ± 3.5¶
5	AOM + 0.1% EGCG	11	40.2 ± 4.4‡	12.4 ± 0.5	55.9 ± 10.3§	13.5 ± 4.6‖	5.7 ± 2.6¶

Abbreviation: AOM, azoxymethane.
*"Large ACFs" are ACFs with four or more aberrant crypts.
†Mean ± SD.
‡Significantly different from group 1 ($P < 0.05$).
§Significantly different from group 3 ($P < 0.05$).
‖Significantly different from group 3 ($P < 0.001$).
¶Significantly different from group 3 ($P < 0.01$).

Effects of EGCG on the serum levels of insulin, IGF-I, and IGFBP-3 and on the expression levels of IGF-IR, p-IGF-IR, p-GSK-3β, β-catenin, COX-2, and cyclin D1 proteins in azoxymethane-treated db/db mice

The effects of EGCG on the serum levels of insulin, IGF-I, and IGFBP-3 and on the activation of IGF-IR protein were next examined because hyperinsulinemia and up-regulation of IGF/IGF-IR axis play a role in obesity-related colorectal cancer development (Fig. 2; refs. 6, 11–13, 26). We found that drinking water containing EGCG caused a significant decrease in the serum levels of both insulin and IGF-I (Fig. 3A and B). On the other hand, drinking with 0.1% EGCG caused a significant increase in the level of IGFBP-3 in azoxymethane-treated mice (Fig. 3C).

As shown in Fig. 3D, a Western blot analyses showed that drinking water with EGCG markedly decreased the levels of IGF-IR and the phosphorylated (i.e., activated) form of IGF-IR (p-IGF-IR) proteins in the colonic mucosa of azoxymethane-treated mice. In addition, there was also a marked decrease in the expression levels of the phosphorylated (i.e., inactivated) form of GSK-3β (p-GSK-3β), β-catenin, COX-2, and cyclin D1, a downstream molecule in the Wnt/β-catenin signaling pathway (33), after drinking water containing EGCG (Fig. 3D). The finding that EGCG caused a decrease in the expression of COX-2 protein seems to be of interest because an increase in this protein expression has been reported to play a significant role in colorectal cancer development and therefore might be one of the targets for chemoprevention of colorectal cancer (34).

Discussion

The present study clearly indicated that EGCG administration in drinking water effectively suppressed the development of putative precursor lesions (ACF and BCACs) of colonic adenocarcinoma, thus suggesting the inhibitory effects of EGCG on the early phase of obesity-related mouse colon carcinogenesis initiated with azoxymethane (Table 1). In addition, this suppressing effect of EGCG was associated with the improvement of hyperlipidemia (Table 2) and hyperinsulinemia (Fig. 3A). Although green tea consumption is not associated with decreased risk of colorectal cancer in general epidemiologic studies (35), our findings may suggest that EGCG might effectively prevent colorectal cancer development, at least, in humans with high risk for developing colorectal cancer, such as obese people.

What is the target of EGCG for its chemopreventive effects on obesity-related cancers? A previous study indicated that EGCG suppressed biosyntheses of lipids and cell proliferation in human breast cancer MCF-7 cells by inhibiting the epidermal growth factor receptor/phosphatidylinositol 3-kinase/Akt signaling pathway (36). EGCG seems to down-regulate adipocyte differentiation by inhibiting activation of extracellular signal-regulated kinase protein (37). With respect to targets at RTKs and its downstream signaling pathways, EGCG also inhibits activation of the epidermal growth factor receptor family of RTKs and its downstream signaling molecules, including extracellular signal-regulated kinase and Akt proteins, thereby inhibiting the growth of colon cancer cells (15, 20, 21). These findings suggest that EGCG exerts both anticancer and antiobesity effects, at least in part, by inhibiting the activation of some types of RTKs and their downstream molecules. We are now trying to reveal evidence that EGCG prevents obesity-related colorectal carcinogenesis by improving such metabolic abnormalities as hyperlipidemia and hyperinsulinemia, while focusing on the effects of EGCG with regard to specific RTKs in an ongoing study.

IGF-IR is also a membrane-associated RTK. It is widely appreciated that the IGF/IGF-IR system is involved in colorectal carcinogenesis and might play a critical role as a molecular target with respect to the prevention and treatment of colorectal cancer (11–13). Ealey et al. (11) showed that both IGF-I and insulin are important promoter of colon cancer development using liver-specific IGF-I–deficient mice. Blockade of the IGF/IGF-IR axis by soluble IGF-IR inhibits IGF-I–induced activation of Akt and growth of colon cancer xenografts *in vivo* (38). The results of the current study indicate that EGCG inhibits the activation of IGF-IR by decreasing the serum levels of the ligand for this receptor, IGF-I, but also increases the levels of IGFBP-3, which can sequester IGF-I and thereby inhibits its activity as an agonist (Fig. 3B and C; refs. 12, 13). Similar effects by green tea catechins are also reported in a prostate cancer chemoprevention study using transgenic mice (39). The finding that a low concentration (0.01%) of EGCG is sufficient to decrease the serum levels of IGF-I (Fig. 3B) might be explained by the direct effect of EGCG on the transcriptional activity of this molecule. Namely, EGCG directly inhibits the expression of IGF-I/2 mRNAs by inhibiting the Ras/mitogen-activated protein kinase and phosphatidylinositol 3-kinase/

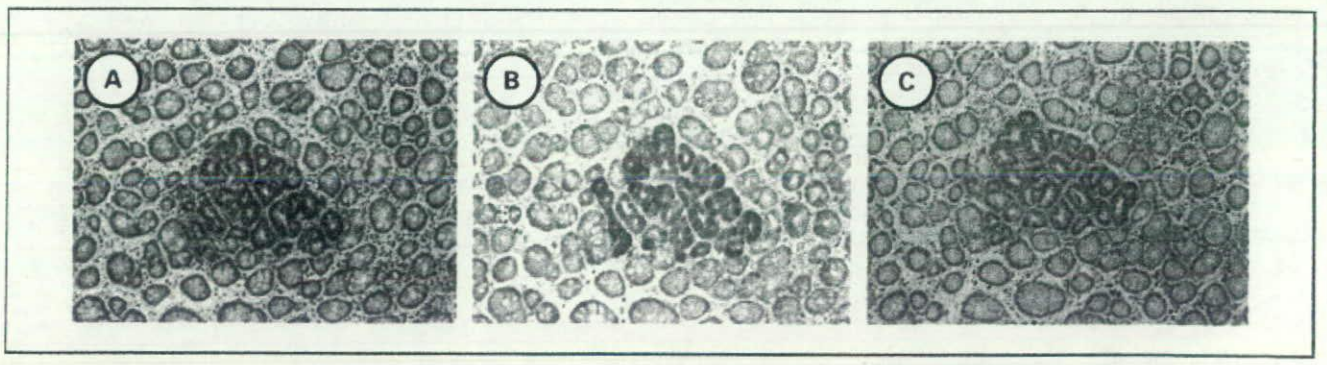

Fig. 2. Histopathology and immunohistochemical expression of β-catenin and IGF-IR in BCACs. *A*, a representative photograph of BCACs induced by azoxymethane in *db/db* mice (H&E staining). The epithelium had basophilic cytoplasm and hyperchromatic nuclei. *B*, immunohistochemistry of β-catenin protein in BCACs. The localization of the accumulated β-catenin protein is apparent in the cytoplasm and nucleus of atypical cryptal cells. *C*, immunohistochemical pattern of IGF-IR protein in BCACs. Strong cytoplasmic expression of the IGF-IR protein is observed in BCACs.

Table 2. Serum triglyceride, total cholesterol, and leptin levels of the experimental mice

Group no.	Treatment	No. mice	Triglyceride (mg/dL)	Total cholesterol (mg/dL)	Leptin (ng/mL)
1	None	6	556.2 ± 27.7*	207.2 ± 22.5	270.0 ± 13.9
2	0.1% EGCG	6	353.4 ± 49.9†	204.2 ± 4.9	248.8 ± 21.0
3	AOM alone	11	244.8 ± 33.8†	176.4 ± 18.8†	273.8 ± 7.2
4	AOM + 0.01% EGCG	11	146.2 ± 39.4‡	131.8 ± 13.6‡	228.0 ± 8.8‡
5	AOM + 0.1% EGCG	11	158.6 ± 39.8‡	127.4 ± 16.1‡	224.3 ± 12.3‡

*Mean ± SD.
†Significantly different from group 1 ($P < 0.01$).
‡Significantly different from group 3 ($P < 0.01$).

Akt signaling pathways in human colorectal cancer and liver cancer cells (22, 23). EGCG also increased the expression of IGFBP-3 protein in these cancer cells. However, the production of IGFBP-3 might be a secondary effect because EGCG indirectly induces this protein by controlling the expression of other molecules, including transforming growth factor-β and matrix metalloproteinase-7 and matrix metalloproteinase-9 (22). Therefore, a high concentration (0.1%) of EGCG might be required to increase the serum levels of IGFBP-3 in the current study (Fig. 3C).

Among the IGF-IR–related downstream molecules, GSK-3β, which is phosphorylated by phosphatidylinositol 3-kinase/Akt, is considered as a key kinase for colorectal cancer development because inactivation of GSK-3β leads to dissociate the adenomatous polyposis coli/axin/β-catenin complex and cytosolic β-catenin accumulation (40). The stability of β-catenin protein caused by IGF-I stimulation results in an enhanced transcriptional activity of target genes in combination with the inhibitor of GSK-3β (31). Free accumulated β-catenin translocates into nucleus and forms a complex with transcription factor "T-cell factor," thereby activating the transcription of target genes, including cyclin D1, and thus contributes to colorectal cancer progression (33). These reports might explain the current finding that the expression of the IGF-IR protein was relatively strong in the cytoplasm of atypical cells in BCACs compared with the surrounding cryptal cells (Fig. 2C), and that the p-IGF-IR, p-GSK-3β, β-catenin, and cyclin D1 proteins were constitutively overexpressed in the colonic mucosa of azoxymethane-treated mice (Fig. 3D).

In addition, recent studies have revealed that COX-2, one of the main mediators in the inflammatory signaling pathway, and its product prostaglandin E_2 (PGE_2), both of which are involved in colorectal cancer development, also stimulate the β-catenin/T-cell factor–mediated pathway in colorectal cancer development (41, 42). PGE_2 increases the p-GSK-3β and consequently accumulates β-catenin, thereby activating the β-catenin/T-cell factor–dependent transcription and increasing the expression of cyclin D1 in colon cancer cells (43). In the present study, blockade of IGF/IGF-IR axis by EGCG caused a decrease in the p-GSK-3β, β-catenin, cyclin D1, and COX-2 proteins in the colonic mucosa (Fig. 3D). EGCG also inhibits the expression of COX-2 and production of PGE_2 both in colorectal cancer cells (22) and in an inflammation-related mice colon carcinogenesis (29). Such findings seem to be significant because obesity-related insulin resistance is associated with a state of chronic inflammation, thus promoting carcinogenesis in certain tissues, including the colon (44, 45).

Leptin is considered to be involved in intestinal carcinogenesis. However, the data showing whether leptin inhibits or promotes the carcinogenesis remain contradictory. Leptin inhibited azoxymethane-induced precancerous lesions in the rat colon (46). Leptin also did not promote the growth of colon cancer xenografts in nude mice or intestinal tumorigenesis in Apc(Min/+) mice (47). On the other hand, in the present study, EGCG decreased the serum leptin level (Table 2) and this is regarded as one of the anticarcinogenic mechanisms of EGCG. In addition, we recently reported that the serum leptin level in mice of colitis-related colorectal cancer model induced by azoxymethane plus dextran sulfate sodium was six times higher than that in untreated mice. In this study, Nobiletin, a citrus flavonoid, abolished colonic malignancy and notably decreased the serum leptin level by 75% (48). These findings, therefore, suggest that a higher serum leptin level exerts tumor promotion effect, at least in part, in obesity- and inflammation-related colorectal cancer.

We should emphasize that not only a high (0.1%) but also a low (0.01%) concentration of EGCG similarly decreased the development of colonic premalignant lesions (Table 1) by improving the obesity-related metabolic abnormalities to the same extent (Table 2; Fig. 3). Similar results which show that drinking low as well as high doses of EGCG could inhibit the development of adenocarcinoma to the same extents are also observed in inflammation-related mouse colon carcinogenesis (29). These reports indicate that a low dose of EGCG (0.01%) is sufficient to prevent the development of colon tumor and suggest the possibility that EGCG dose thus can be further decreased, although the feeding protocol of EGCG at a high dose (0.1%) mimics an approximate consumption of six cups of green tea per day by an average adult human and has been used in mice in many prior chemopreventive studies (14, 39, 49). These findings, therefore, might be more significant when considering the clinical use of this agent because a lower dose is more acceptable for administration to patients.

Finally, when checking the potency of ACF reduction by many chemopreventive agents in the Chemoprevention Database,[5] that of EGCG was below the average (average database potency around 2.0, compared with EGCG 1.48 = 83.2/57.1 or

[5] http://www.inra.fr/reseau-nacre/sci-memb/corpet/indexan.html

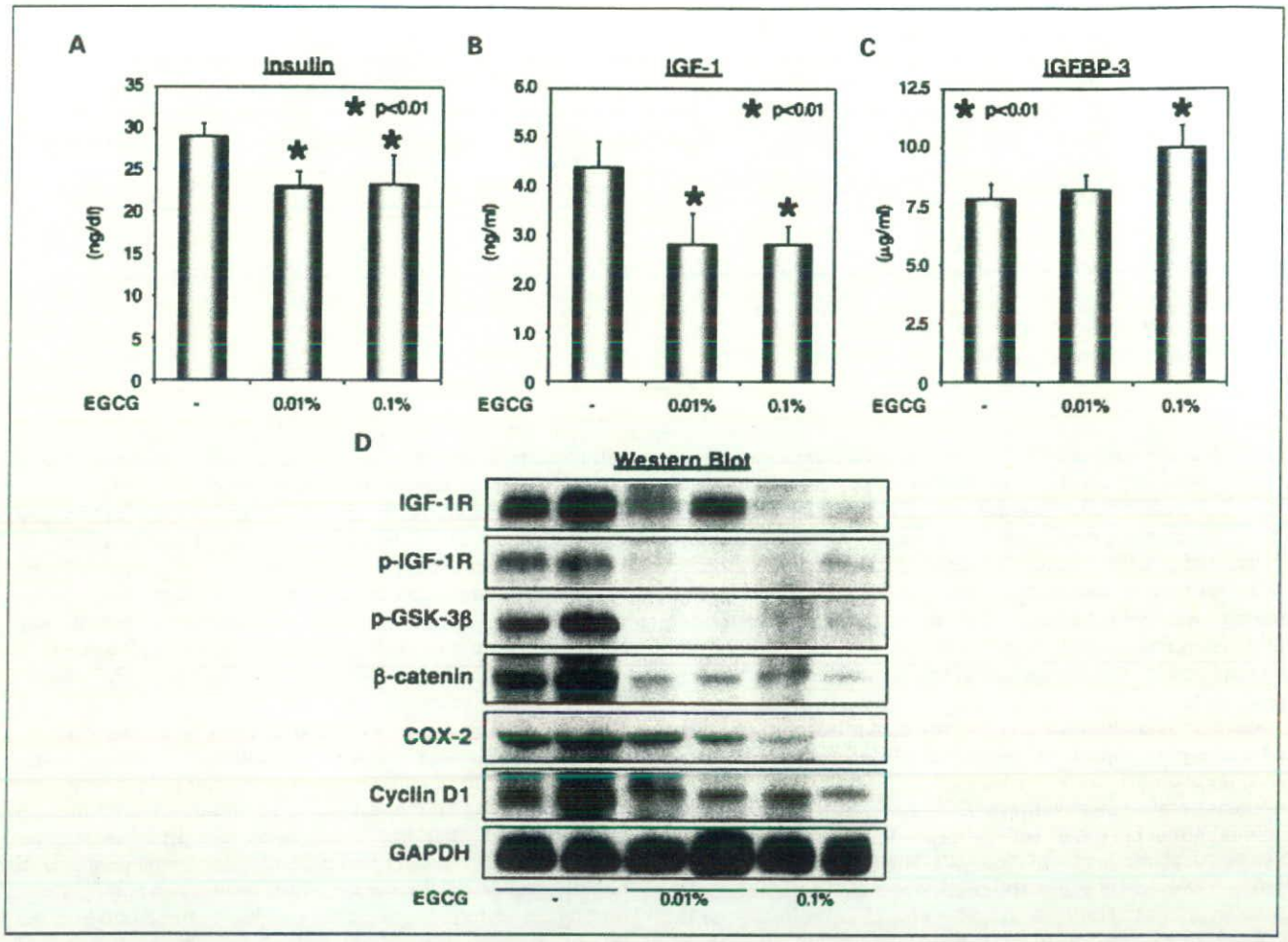

Fig. 3. The effect of EGCG on serum levels of insulin, IGF-I, and IGFBP-3 and on activation of the IGF-IR and its downstream signaling molecules in azoxymethane-treated *db/db* mice. The serum concentrations of insulin (*A*), IGF-I (*B*), and IGFBP-3 (*C*) were measured by an enzyme immunoassay. The asterisks indicate a significant difference ($P < 0.01$) between the control azoxymethane-treated group and the EGCG-treated groups. *Bars*, SD of triplicate assays. *D*, total proteins were extracted from the scraped colonic mucosa of azoxymethane-treated mice and equivalent amounts of proteins were examined by a Western blot analysis, as described in Materials and Methods. Two lanes represent protein samples from two different mice in each group (groups 3–5). Repeat Western blots gave similar results.

83.2/55.9; see Table 1). However, we should evaluate the inhibitory effects of EGCG with regard to the actual development of colonic tumors. In conclusion, the prevention of colorectal cancer by targeting the dysregulation of energy homeostasis might be one of the promising strategies for obese patients who are at increased risks of colorectal cancer. EGCG seems to be a potentially effective and critical candidate for this purpose because this agent can exert a depressant effect on the IGF/IGF-IR and COX-2/PGE_2 axes, and both of these axes are critical targets for colorectal cancer chemoprevention (12, 13, 34).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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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 azoxymetahen (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.

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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

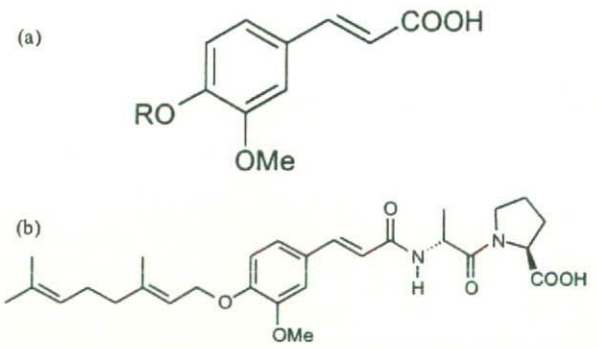

FIG. 1. Chemical structure of (a) ferulic acid. R = H and 3-(4-geranyloxy-3′-methoxyphenyl)-2-*trans*-propenoic acid, R = geranyl, and (b) 3-(4′-geranyloxy-3′-methoxyphenyl)-2-trans-propenoyl-L-alanyl-L-proline (GAP, molecular weight = 498.62).

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 collinin 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′-geranoxy-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′-geranoxy-3′-methoxyphenol)-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, Fig. 1b). This novel prodrug of 4′-geranyloxy-ferulic acid was structurally built to be hydrolized by intestinal angiotensin-converting enzyme; this enzyme is an exopeptidase that is quite abundant in the external side of the brush border of the epithelium of the small intestine, and its specificity is to hydrolize the last peptidic link in tripeptides in which +-Ala (or Gly) and L-pro occupy the second-last and last positions, respectively. Based on features of this prodrug, 3-(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 azoxymetahen (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%), lightning (12-h light/dark cycle), and temperature (23°C ± 2°C). They were quarantined for 7 days after arrival and randomized by body

weight into experimental and control groups. A colonic carcinogen 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 diets 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 handling 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 administrated 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 CO_2 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. Colonic tumors were diagnosed according to the Ward's (37) description. In brief, if the tumors cells with tubular formation invaded the depth of submucosa, the tumor was diagnosed as adenocarcinoma. When the tumors cells with glandular structure did not invade the submucosa and compressed the surrounding crypts, the tumor was diagnosed as adenoma.

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 moderate inflammation in the mucosa; Grade 3, loss of the entire crypts with severe inflammation in the mucosa and submucosa but with retainment of the surface epithelium; and Grade 4, presence of mucosal ulcer with severe inflammation (infiltration of neutrophils, lymphocytes, and plasma cells) in the mucosa, submucosa, 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 determined 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. Randomly 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 solutions 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 antibody (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 Laboratories). At the last step, the sections were counterstained with hematoxylin. As a negative control, omission of the primary antibody was used. Two observers (T. Tanaka and S. Sugie) were

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 Softwear, 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.

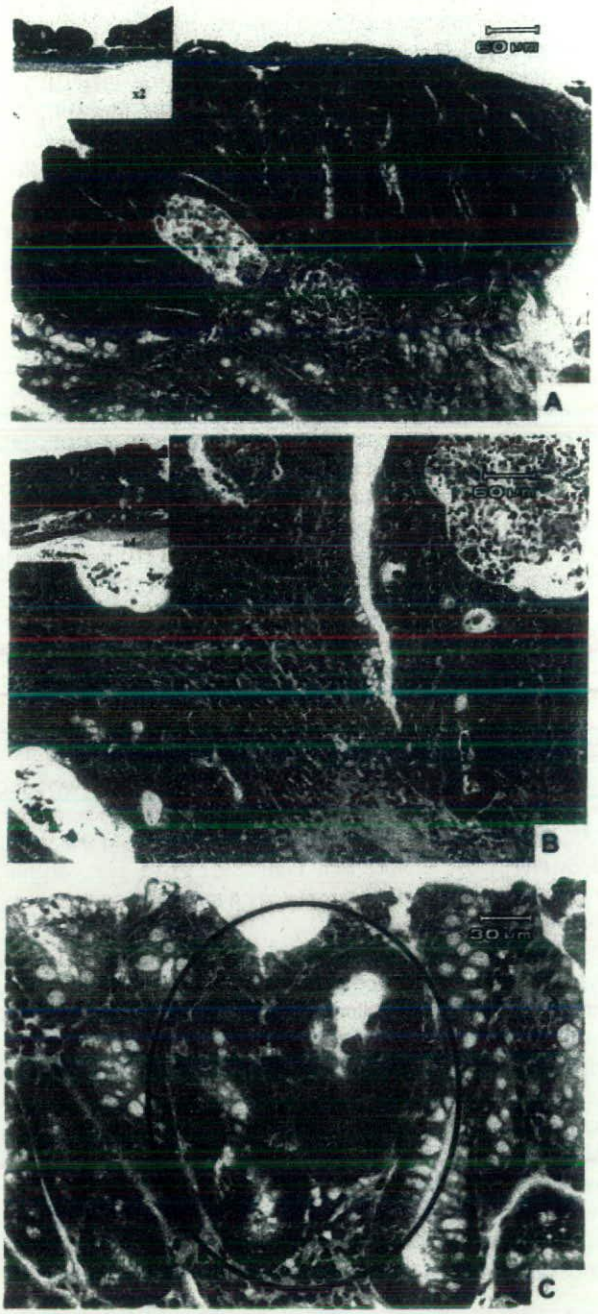

FIG. 2. Representative colonic lesions induced by azoxymetahen/dextran sodium sulfate in mice (Group 1): A: a tubular adenoma, B: a tubular adenocarcinoma with moderately differentiated, and C: dysplastic crypts (circled). Photos inserted in Fig. 2A and 2B are low power of views for each lesion (original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

TABLE 1
Incidence and Multiplicity of Colonic Tumors[a]

Group	Treatment	Incidence (No. of Mice With Tumors)			Multiplicity (No. of Tumors/Mouse)[b]		
		Total	AD	ADC	Total	AD	ADC
1	AOM/DSS (20)	20 (100%)	19 (95%)	19 (95%)	5.60 ± 4.81	2.50 ± 2.37	3.10 ± 3.06
2	AOM/DSS → 0.01% GAP (15)	10 (67%)[c]	8 (53%)[d]	9 (60%)[e]	2.33 ± 2.12[f]	1.20 ± 1.27	1.13 ± 1.13[f]
3	AOM/DSS → 0.05% GAP (15)	10 (67%)[c]	8 (53%)[d]	8 (53%)[d]	2.00 ± 2.10[f]	1.20 ± 1.37	0.80 ± 1.08[g]
4	0.05% GAP (5)	0 (0%)	0(0%)	0(0%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5	None (5)	0 (0%)	0(0%)	0(0%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

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

[b] Mean ± SD.

[c] Significantly different from the AOM/DSS group by Fisher's exact probability test, $P = 0.0002$.

[d] Significantly different from the AOM/DSS group by Fisher's exact probability test, $P = 0.0057$.

[e] Significantly different from the AOM/DSS group by Fisher's exact probability test, $P = 0.0158$).

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

[g] 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 colonic 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 colonic 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.

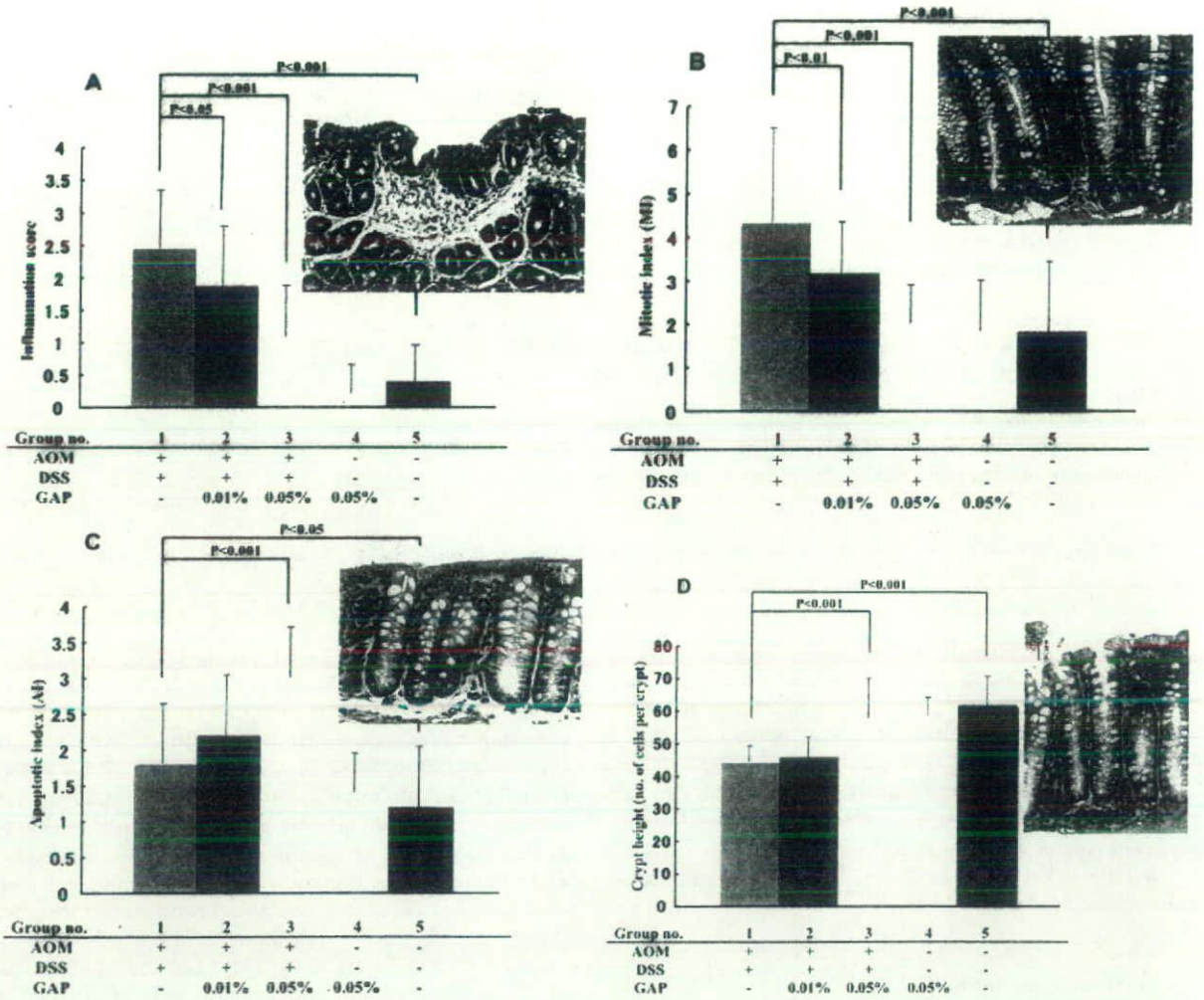

FIG. 3. A: Inflammation score (mean ± SD) of colon from each group. Photo shows inflammation score, Grade 2, in the colon of a mouse from Group 1; hematoxylin and eosin (H & E) stain; a bar inserted indicates magnification (μm). B: Mitotic index (MI, mean ± SD) of crypts of each group. For Fig. 3B photos, 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, azoxymetahen; DSS, dextran sodium sulfate; GAP, 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans*-propenoyl-L-alanyl-L-proline.

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

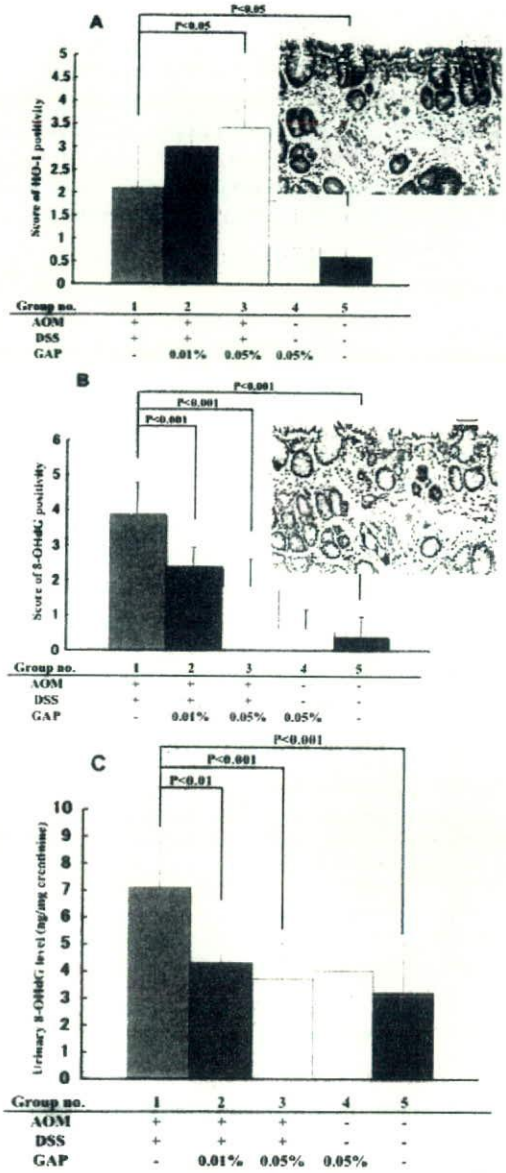

FIG. 4. A: Score (mean ± SD) of heme oxygenase (HO)-1 immunoreactivity. Photo shows strong HO-1 immunoreactivity (Grade 2) of colonic mucosa (same as an inset in Fig. 4) from a mouse of Group 1. Strong positive reaction is present in cryptal cells and inflammatory cells infiltrated into the inflamed 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 carcinogenesis 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-dficient 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: ursodeoxycholic acid (29) > nimesulide (50) > collonin (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.

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Zerumbone, a tropical ginger sesquiterpene, inhibits colon and lung carcinogenesis in mice

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Zerumbone (ZER), present in subtropical ginger *Zingiber zerumbet* Smith, possesses anti-growth and anti-inflammatory properties in several human cancer cell lines. ZER also down-regulates the cyclooxygenase-2 and inducible nitric oxide synthase expression *via* modulation of nuclear factor (NF)-κB activation in cell culture systems. These findings led us to investigate whether ZER is able to inhibit carcinogenesis in the colon and lung, using 2 different preclinical mouse models. In Exp. 1, a total of 85 male ICR mice were initiated using a single intraperitoneal (i.p.) injection with azoxymethane (AOM, 10 mg/kg bw) and promoted by 1.5% dextran sulfate sodium (DSS) in drinking water for 7 days for rapid induction of colonic neoplasms. Animals were then fed the diet containing 100, 250 or 500 ppm ZER for 17 weeks. In Exp. 2, a total of 50 female A/J mice were given a single i.p. injection of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (10 μmol/mouse) to induce lung proliferative lesions. They were then fed the diet mixed with 100, 250 or 500 ppm ZER for 21 weeks. At the termination of the experiments (wk 20 of Exp. 1 and wk 22 of Exp. 2), all animals were subjected to complete necropsy examination to determine the pathological lesions in both tissues. Oral administration of ZER at 100, 250 and 500 ppm significantly inhibited the multiplicity of colonic adenocarcinomas. The treatment also suppressed colonic inflammation. In the lung carcinogenesis, ZER feeding at 250 and 500 ppm significantly inhibited the multiplicity of lung adenomas in a dose-dependent manner. Feeding with ZER resulted in inhibition of proliferation, induction of apoptosis, and suppression of NFκB and heme oxygenase (HO)-1 expression in tumors developed in both tissues. Our findings suggest that dietary administration of ZER effectively suppresses mouse colon and lung carcinogenesis through multiple modulatory mechanisms of growth, apoptosis, inflammation and expression of NFκB and HO-1 that are involved in carcinogenesis in the colon and lung.

Key words: zerumbone; colon; lung; carcinogenesis; chemoprevention

A sesquiterpenoid, zerumbone (ZER), is a major constituent of the subtropical ginger plant *Zingiber zerumbet* Smith. The essential oil of the rhizomes contains large amount of ZER and is used as an anti-inflammatory medicine.[1] Recent studies revealed several biological properties of ZER that may be responsible for inhibition of carcinognesis. They include suppression of skin tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein-Barr virus activation in Raji cells,[1] inhibition of free radical generation, inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expression, inhibition of tumor necrosis factor (TNF)-α-release in activated leukocytes and induction of apoptosis in human colonic adenocarcinoma (ADC) cell lines.[2] *In vivo* studies demonstrated that dietary feeding with ZER markedly suppressed dextran sulfate sodium (DSS)-induced acute colitis in mice[3] and a putative precursor lesions for colonic ADC, aberrant crypt foci (ACF), produced by azoxymethane (AOM) in rat colon.[4] The findings were accompanied by reductions of prostaglandin E_2 and COX-2 protein expression in colonic mucosa.[3,4] Additionally, ZER suppresses the combined lipopolysaccharide- and interferon-γ-induced IκB protein degradation in macrophages.[5] More recently, Takada *et al.*[6] reported that ZER suppresses nuclear factor (NF)-κB activation induced by TNF, okadaic acid, cigarette smoke condensate, TPA and H_2O_2.

Colorectal cancer (CRC) and lung cancer are major epithelial malignancies and both are increasing in developed countries. An association between inflammation and cancer has long been suspected[7] and inflammatory condition is a risk for cancer development in colon and lung.[8–12] A representative example is that inflamed colon has a high risk for CRC development. In patients with inflammatory bowel disease, including ulcerative colitis and Crohn's disease, the risk of CRC development is greater than in the general population.[13] Smoking is a risk factor of development of different types of cancer in different tissues, including lung[14] and colon.[15] Also, inflammation caused by tobacco enhances lung carcinogenesis.[11] Despite well-developed diagnostic and therapeutic techniques, and novel anti-cancer drugs against both malignancies have been introduced, mortality rates of CRC and lung cancer have not remarkably been improved. Therefore, we need some new weapons and strategies for fighting against these malignancies. Cancer chemoprevention is one of such strategies. For clinical use of candidate cancer chemopreventive agents, they need to be determined preclinical efficacy using appropriate animal carcinogenesis models. As to inflammation-associated colon carcinogenesis, we have developed a mouse model utilizing a colon carcinogen AOM and a colitis-inducing agent DSS.[16] 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced mouse lung tumorigenesis model is frequently used for carcinogenesis and chemoprevention studies, as NNK is a tobacco-specific carcinogenic nitrosamine, which derived from nicotine.[14,17] Hecht *et al.*[18] developed a relatively rapid single-dose model for induction of lung adenomas (ADs) in female A/J mice initiated with NNK. The fact that nonsteroidal anti-inflammatory drugs can inhibit NNK-induced lung tumors[19] suggests that inflammation is involved in NNK-induced lung tumorigenesis.

In the current study, we investigated the chemopreventive ability of ZER in colon and lung carcinogenesis using a mouse colitis-related CRC model[16] and a NNK-induced mouse lung carcinogenesis model.[18] Also, the effects of ZER on the immunohistochemical

Abbreviations: ACF, aberrant crypt foci; AD, adenoma; ADC, adenocarcinoma; AOM, azoxymethane; ARE, antioxidant response element; CRC, colorectal cancer; DAB, 3,3'-diaminobenzidine; DSS, dextran sulfate sodium; GPx, γ-glutamylcystein glutathione peroxidase; HO, heme oxygenase; H&E, hematoxylin and eosin; HP, hyperplasia; iNOS, inducible nitric oxide synthase; MnSOD, manganese superoxide dismutase; NF-κB, nuclear factor-kappaB; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NrF, nuclear factor-erythroid 2-related factor; PCNA, proliferating cell nuclear antigen (PCNA); TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TUNEL, TdT-mediated dUTP nick-end labeling; ZER, zerumbone.

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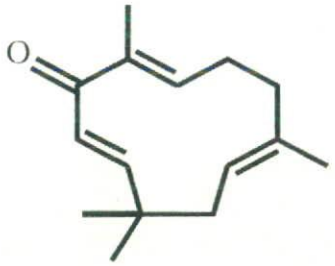

FIGURE 1 – Chemical structure of zerumbone (2,6,9,9-tetramethyl-[2*E*,6*E*,10*E*]-cycloundeca-2,6-10-trien-1-one, MW 218, purity>95%).

expression of heme oxygenase (HO)-1 and NFκB in colonic and lung neoplasms were examined, since the expression of these molecules are involved in carcinogenesis and inflammation[20–22] and certain suppressors of these molecules' expression are candidates for cancer chemopreventive agents.[23,24]

Material and methods

Chemicals

ZER (2,6,9,9-tetramethyl-[2*E*,6*E*,10*E*]-cycloundeca-2,6-10-trien-1-one, MW 218, purity > 95%, Fig. 1) was isolated and purified from a chloroform extract of the rhizomes of Z. *zerumbet* Smith, as previously reported.[1]

AOM was purchased from SIGMA-ALDRICH (St. Louis, MO). DSS with a molecular weight of 36,000–50,000 (Cat. no. 160110) was obtained from MP Biomedicals, LLC (Aurora, OH). DSS for induction of colitis was dissolved in water at a concentration of 1.5% (*w/v*) just before it was used. NNK was obtained from Toronto Research Chemical Inc. (Ontario, Canada).

Animals and diets

The animal experiments were approved by the Committee of the Institutional Animal Experiments and were conducted at the Kanazawa Medical University Animal Facility under the Institutional Animal Care Guideline. Five-week-old male ICR mice for Exp. 1 (CRC chemoprevention study) and 5-week-old female A/J mice for Exp. 2 (lung cancer chemoprevention study) were purchased from Charles River Laboratories, Inc. (Tokyo, Japan). All animals were housed in plastic cages (5 mice/cages) and had free access to drinking water and a basal diet (CRF-1, Oriental Yeast, Co., Ltd., Tokyo, Japan) *ad libitum*, under controlled conditions of humidity (50 ± 10%), light (12/12 hr light/dark cycle) and temperature (23 ± 2°C). After arrival, they were quarantined for the first 7 days, and then randomized by their body weights into experimental and control groups. Experimental diets were prepared by mixing zerumone in powdered basal diet CRF-1 at dose levels (w/w) of 100, 250 and 500 ppm, based on our previous study.[4] We confirmed that ZER is quite stable in the diets (data not shown). Animals had access to food and water at all times. Cups were replenished with fresh food everyday. All handling and procedures were carried out in accordance with the Institutional Animal Care Guidelines.

Exp. 1: Effect of ZER on AOM/DSS-induced colon carcinogenesis in mice

A total of 85 male ICR mice were divided into 7 experimental and control groups. They were given a single intraperitoneal (i.p.) injection with AOM (10 mg/kg, bw). Starting 7 days after the AOM injection, they received 1.5% (*w/v*) DSS in the drinking water for 7 days. Seven days later, ZER-containing diets were started and the mice continued on these experimental diets for 17 weeks. Experimental groups included group 1 (n = 20) that received AOM and DSS; groups 2–4 (n = 15 for each group) were treated with AOM, DSS and ZER (100 ppm for group 2, 250 ppm for group 3, and 500 ppm for group 4) in diet; group 5 (n = 5) was treated with DSS and ZER (500 ppm); group 6 (n = 5) was treated with DSS alone; group 7 (n = 5) was given the diet containing 500 ppm ZER alone; group 8 was an untreated group. All animals were subjected to a complete gross necropsy examination at the time of euthanasia (wk 20) to determine the incidence and multiplicity of tumors in the large bowel. At sacrifice, the large bowel was removed and macroscopically inspected for the presence of tumors. After measuring the colon length (from the ileocecal junction to the anal verge), large bowels were cut open longitudinally along the main axis and gently washed with saline. Fifteen mice in group 1, 10 each from groups 2–4, and 2 each from groups 5–8 were randomly selected for histopathology. They were cut along the vertical axis and then fixed in 10% buffered formalin for at least 24 hr. The colons of remaining animals were saved for future molecular analysis. Histopathological examination was performed on hematoxylin and eosin (H&E)-stained sections made from paraffin-embedded blocks. Colitis was recorded and scored according to the following morphological criteria described by Cooper *et al.*[25]: Grade 0, normal colonic mucosa; Grade 1, shortening and loss of the basal one-third of the actual crypts with mild inflammation and edema in the mucosa: Grade 2, loss of the basal two-thirds of the crypts with moderate inflammation in the mucosa; Grade 3, loss of all crypts with severe inflammation in the mucosa, but with the surface epithelium still remaining; and Grade 4, loss of all crypts and the surface epithelium with severe inflammation in the mucosa, muscularis propria and submucosa. Colonic tumors were diagnosed according to the criteria described by Ward.[26]

Exp. 2: Effect of ZER on NNK-induced lung carcinogenesis

To determine the effect of ZER on lung carcinogenesis, a total of 50 female A/J mice were divided into 5 experimental and control groups. The mice were given a single i.p. injection with NNK (10 μmol/mouse). Seven days after the injection, they were fed the diets mixed with ZER at 3 different dose levels (100, 250 and 500 ppm) for 21 weeks. Experimental groups were: group 1 (n = 10) given NNK alone; Groups 2 (n = 10) given NNK and 100 ppm ZER; group 3 received NNK and 250 ppm ZER; group 4 (n = 10) treated with NNK and 500 ppm ZER; group 5 received 500 ppm ZER alone; and group 6 was an untreated control. All mice were subjected to a complete gross necropsy examination at the time of euthanasia (wk 22) to histopathologically investigate proliferative lesions, alveolar cell hyperplasia (HP) and neoplasms in the lung. At sacrifice, all lobes of the lung were removed, weighed and macroscopically inspected for the presence of tumors and/or nodules. They were fixed in 10% buffered formalin for at least 24 hr for histopathological examination on H&E-stained sections made from paraffin-embedded blocks. Lung proliferative lesions including HP and neoplasms were diagnosed according to the criteria Nikitin *et al.*[27]

Immunohistochemistry of proliferating cell nuclear antigen (PCNA), apoptotic nuclei, NF-κB and HO-1

Immunohistochemical analysis for the PCNA, NF-κB and HO-1-positive neoplastic cells in the colon and lung was performed on 4-μm-thick paraffin-embedded sections by the labeled streptavidin biotin method using a LSAB KIT (DAKO Japan, Kyoto, Japan), with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65°C, de-paraffinized in xylene and rehydrated through grade ethanol at room temperature. Tris HCl buffer (0.05 M, pH 7.6) was used to prepare the solutions and was used for washes between the various steps. Incubations were performed in a humidified chamber.

The sections were treated for 40 min at room temperature with 2% bovine serum albumin, and incubated overnight at 4°C with primary antibodies. The primary antibodies included anti-human PCNA mouse monoclonal antibody (DAKO #U 7032, 1:1000 dilution; DAKO Japan), anti-HO-1 rabbit polyclonal antibody (OSA-150C, diluted 1:200, StressGen Biotechnologies, Ann Arbor, MI)

TABLE I – EFFECTS OF DIETARY ZEROMBONE ON COLONIC INFLAMMATION AND DEVELOPMENT OF MUCOSAL ULCER AND HIGH GRADE DYSPLASIA (EXP. 1)

Group no.	Treatment	No. of mice examined	Inflammatory score	Number of mucosal ulcer	No. of dysplasia (high grade)
1	AOM[1]/DSS	15	1.80 ± 0.77[2]	2.00 ± 1.00	1.80 ± 1.01
2	AOM/DSS + 100 ppm ZER	10	0.80 ± 0.63[3]	1.20 ± 0.63	1.70 ± 1.49
3	AOM/DSS + 250 ppm ZER	10	0.50 ± 0.71[4]	0.60 ± 0.84[3]	1.00 ± 1.63
4	AOM/DSS + 500 ppm ZER	10	0.30 ± 0.48[4]	0.50 ± 0.71[4]	1.10 ± 1.66
5	DSS + 500 ppm ZER	5	2.50 ± 0.71	1.50 ± 0.71	0
6	DSS	5	4.00 ± 0.00	4.50 ± 0.71	0
7	500 ppm ZER	5	0	0	0
8	Untreated	5	0	0	0

[1]AOM, azoxymethane; DSS, dextran sulfate sodium; and ZER, zerumbone.–[2]Mean ± SD.–[3,4]Significantly different from the AOM/DSS group (group 1) by Tukey-Kramer multiple comparison posttest ([3]$p < 0.01$ and [4]$p < 0.001$).

and anti-NF-κB p50 (H-119) rabbit polyclonal antibody (sc-7178, diluted 1: 500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). These antibodies were applied to the sections according to the manufacturer's protocol. To reduce the nonspecific staining of mouse tissue by a mouse antibodies, a Mouse On Mouse IgG blocking reagent (MKB-2213, Vector Laboratories, Burlingame, CA) was applied for 1 hr. Horseradish peroxidase activity was visualized by treatment with H_2O_2 and 3,3'-diaminobenzidine (DAB) for 5 min. At the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck, Tokyo, Japan). For each case, negative controls were performed on serial sections.

Levels of apoptosis in tumor tissues were determined by the TdT-mediated dUTP nick-end labeling (TUNEL) method. Four-micrometer formalin-fixed, paraffin-embedded tissue sections of colonic ADCs and lung ADs from groups 1 through 4 of Exp. 1 and 2 were processed according to manufacturer's instructions using Apoptosis in situ Detection Kit Wako (Cat. No. 298-60201, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The kit is based on TUNEL procedure. Appropriate positive and negative controls for determining the specificity of staining were generated. Negative controls were processed in the absence of the terminal deoxynucleotidyl transferase (TdT) enzyme in the reaction buffer. Sections of tissue digested with nuclease enzyme and colon lymphoid nodules, which are known to exhibit high rates of apoptosis, were used as positive controls. Color was developed with the peroxidase substrate DAB, and sections were counterstained with hematoxylin.

The numbers of nuclei with positive reactivity for PCNA- and TUNEL-immunohistochemistry were counted in a total of 3 × 100 cells in 3 different areas of the tumors, and expressed as percentage (mean ± SD). Intensity and localization of immunoreactivities against the primary antibodies, NF-κB and HO-1, were assessed using a microscope (Olympus BX41, Olympus Optical Co., Tokyo, Japan). Each slide for the immunohistochemical expression of NF-κB p50 and HO-1 was observed with the grading intensity of the immunoreactivity in neoplasms of the large bowel and lung. Two observers (T.T. and T.O.) were unaware of the treatment groups to which the slides 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). Care was taken to exclude the possibility of any inflammatory cells that were mistakenly identified as positive epithelial cells.

Statistical analysis

The incidences among the groups were compared using Chi-square test or Fisher's exact probability test with the GraphPad Instat Software (version 3.05; GraphPad software Inc., San Diego, CA). Other measurements expressing mean ± SD were statistically analyzed using Tukey-Kramer multiple comparison post test (GraphPad Instat version 3.05). Differences were considered statistically significant at $p < 0.05$.

Results

Exp. 1: Effect of ZER on AOM/DSS-induced colon carcinogenesis in mice

General observation. All animals remained healthy throughout the experimental period. At sacrifice, the mean body weight (51.5 ± 6.4 g, $p < 0.01$) and colon length (14.9 ± 0.7 cm, $p < 0.05$) of the DSS alone group (group 6) were significantly higher than those of the AOM/DSS (group 1, 44.6 ± 2.1 g and 13.8 ± 0.3 cm). Other measures (liver, kidney and spleen weights) did not significantly differ among the groups.

The inflammation scores and numbers of mucosal ulcer and high grade dysplasia in the colon. As summarized in Table I, colonic inflammation with or without mucosal ulcer was observed in the mice of groups 1 through 6. The mean score of inflammation and mean number of mucosal ulcer of group 6 (DSS alone) were the highest among the groups. Oral administration of ZER (groups 2–4) significantly and dose-dependently decreased the inflammatory score (group 2: 0.80 ± 0.63, $p < 0.01$; group 3: 0.50 ± 0.71, $p < 0.001$; and group 4 0.30 ± 0.48, $p < 0.001$) as compared to that of the AOM/DSS group (1.80 ± 0.77). Likewise, 250 ppm (0.60 ± 0.84; $p < 0.01$) and 500 ppm ZER (0.50 ± 0.71; $p < 0.001$) significantly lowered the number of mucosal ulcer. ZER feeding lowered the multiplicity of high grade dysplasia, but the differences were insignificant among the groups.

Colonic neoplasms. Macroscopically, colonic neoplasms developed in the mice of groups 1 through 4 with different incidence and multiplicity. The incidences and multiplicities of histopatholgically confirmed colonic tubular AD, ADC (Fig. 2*a*), and total tumors are given in Table II. Group 1 (AOM/DSS group) had a 93% incidence of colon ADC with a multiplicity of 3.87 ± 2.90. The incidences of ADC in groups 2–4 were smaller than that of group 1 and the value (50%, $p = 0.0225$) of group 4 (AOM/DSS + 500 ppm ZER) was significantly lower than group 1. The multiplicities of colonic ADC in group 2 ($p < 0.05$), 3 ($p < 0.01$) and 4 ($p < 0.01$) were also significantly smaller than group 1.

Effects of ZER on proliferation and apoptosis of colonic ADCs. As given in Figure 3, ZER feeding significantly decreased the PCNA-labeling index at 3 doses ($p < 0.001$ for each dose) of cancer cells (Fig. 3*a*) and significantly increased TUNEL-positive apoptotic nuclei ($p < 0.05$ at 100 ppm, and $p < 0.001$ at 250 and 500 ppm) of cancer cells (Fig. 3*b*).

Immunohistochemical scores of NFκB and HO-1 in colonic ADCs. Immunohistochemistry of NFκB revealed that strong reactivity of inflammatory cells in the inflamed colon (Fig. 4*a*) and ADC cells (Fig. 4*b*). Relative weak reactivity against HO-1 antibody was observed inflammatory cells (Fig. 4*c*) and ADC cells (Fig. 4*d*). As illustrated in Figure 3, ZER feeding dose-dependently lowered the immunohistochemical scores of NFκB (Fig. 3*c*) and HO-1 (Fig. 3*d*).

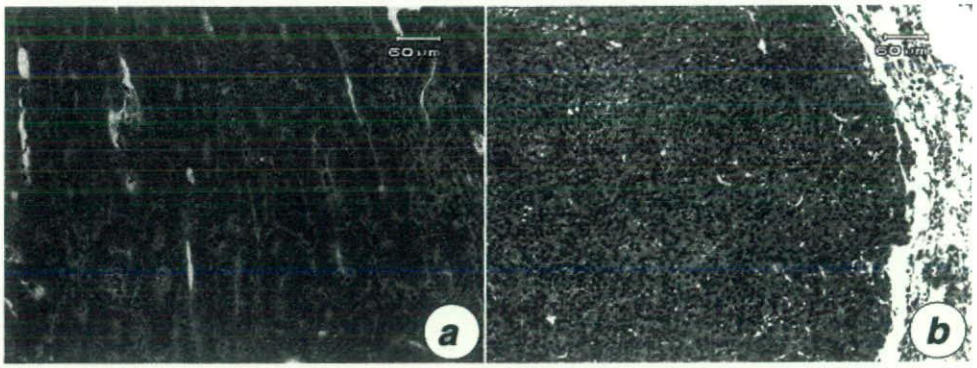

FIGURE 2 – Histopathology of (*a*) colonic tubular ADC induced by AOM/DSS and (b) lung tubular adenoma induced by NNK. Bars in the photos indicate magnification (μm).

TABLE II – EFFECTS OF DIETARY ZERUMBONE ON THE DEVELOPMENT OF COLONIC ADENOMA AND ADENOCARCINOMA (EXP. 1)

Group no.	Treatment	No. of mice examined	Incidence (%)			Multiplicity (no. of tumors/colon)		
			AD	ADC	Total	AD	ADC	Total tumors
1	AOM[1]/DSS	15	60	93	93	1.60 ± 1.55[2]	3.87 ± 2.90	5.47 ± 4.02
2	AOM/DSS + 100 ppm ZER	10	70	70	80	1.60 ± 1.58	1.50 ± 1.51[3]	3.10 ± 2.64
3	AOM/DSS + 250 ppm ZER	10	90	60	90	2.00 ± 1.33	1.20 ± 1.23[4]	3.20 ± 2.39
4	AOM/DSS + 500 ppm ZER	10	50	50[5]	60	0.90 ± 1.10	0.60 ± 0.70[4]	1.50 ± 1.72[3]
5	DSS + 500 ppm ZER	5	0	0	0	0	0	0
6	DSS	5	0	0	0	0	0	0
7	500 ppm ZER	5	0	0	0	0	0	0
8	Untreated	5	0	0	0	0	0	0

[1]AOM, azoxymethane; DSS, dextran sulfate sodium; ZER, zerumbobe; AD, adenoma; and ADC, adenocarcinoma.–[2]Mean ± SD.–[3,4]Significantly different from the AOM/DSS group (group 1) by Tukey-Kramer multiple comparison posttest ([3]$p < 0.05$ and [4]$p < 0.01$).–[5]Significantly different from the AOM/DSS group (group 1) by Fisher's exact probability test ($p = 0.0225$).

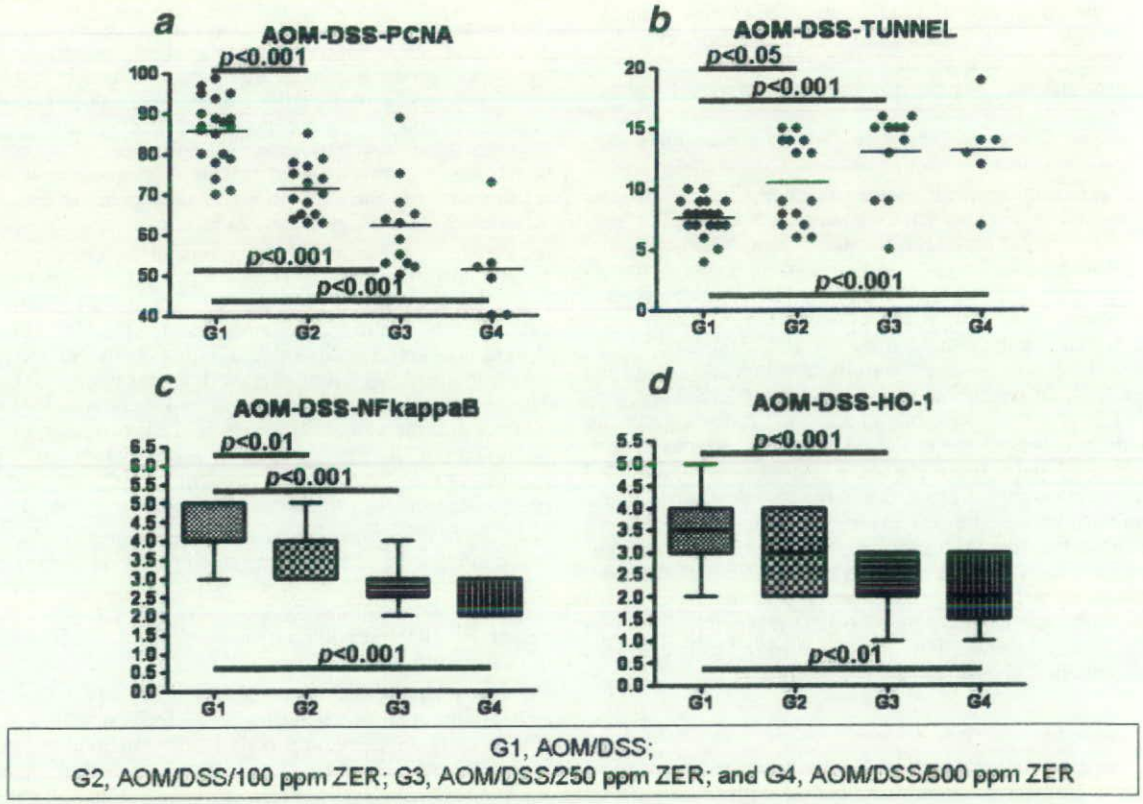

FIGURE 3 – Immunohistochemical scores of (*a*) PCNA-labeling index, (*b*) apoptotic index, (*c*) NF-κB and (*d*) HO-1, which were determined in colonic ADCs developed in mice of groups 1 through 4.