

positive correlation between the positive expression of iNOS and disease progression.

$\beta$ -Catenin functions as a transcriptional activator of the Wnt signaling pathway in embryonic and tumor development (54). In several types of human cancer, mutations in the  $\beta$ -catenin or APC gene cause an accelerated tumor cell proliferation and tumor progression through the transcriptional activation of such target genes as *cyclin D1* (59), with the resulting cytoplasmic/nuclear accumulation of  $\beta$ -catenin (60). In the current study, the immunohistochemical  $\beta$ -catenin expression in the non-lesional tongue epithelium was strictly confined to the cell membranes but not nuclei. Whereas some carcinoma cells preserved a weak membranous expression, the membranous expression of  $\beta$ -catenin decreased while the cytoplasmic/nuclear expression increased in line with the disease progression, and carcinoma cells at the invasion front showed a cytoplasmic/nuclear pattern of  $\beta$ -catenin. We did not perform a gene mutation analysis in the current study, but no mutations of  $\beta$ -catenin gene were observed in the rat tongue carcinomas induced by 4-NQO (32). These findings are in line with those reported in human oral cancers (43). As a result, it may, thus, be possible that molecular events other than mutations in  $\beta$ -catenin and APC are responsible for the activation of the Wnt/ $\beta$ -catenin signaling pathway and the cytoplasmic/nuclear expression of  $\beta$ -catenin in tongue carcinogenesis.

Oral lesions with an aberrant DNA content represent an increased risk of cancer (44,61). The value of the DNA content is useful as an early biomarker of oral cancer (61). In this study, the histological grading of 4-NQO-induced tongue lesions correlated to DNA ploidy. The quantification of the histological evaluation of tissue architecture may show a certain correlation among the degree of dysplasia, COX-2 expression and DNA ploidy (62,63). In the current study, the COX-2 expression was upregulated in DNA aneuploid tongue dysplastic and neoplastic lesions. In contrast, non-lesional 'normal' appearing tongue epithelium specimens that showed a weak COX-2 expression in the basal layer had a diploid DNA content. These findings may indicate that COX-2 is upregulated during malignant transition of the tongue epithelium, and this could be in some manner related to the development of genomic instability (64,65).

Oral cancer is a disfiguring disease that continues to increase in incidence, particularly in the young, and to an extent that cannot be fully explained by an increased exposure to the known risk factors. For such malignancies, a chemopreventive approach to oral cancer most likely should encompass a combination of chemicals targeting the metabolic pathways relevant to oral carcinogenesis. Candidate chemicals include retinoids, selective inhibitors of COX-2 and ligands of PPARs, some of which have been tested for their efficacy by our research group. Although the efficiency of any chemical for chemopreventive use should be assessed through a prospective randomized trial and then evaluated only by a definitive end-point for the prevention of cancer, our Tg rat model using intermediate biomarkers (expression of GST-P, cyclin D1, COX-2, iNOS and  $\beta$ -catenin in tongue lesions and white patches) was, thus, found to be effective for a preclinical evaluation of candidate chemopreventive agents against oral cancer development within a short-term period of time.

In conclusion, we established an animal model of oral SCC using Tg rats and the carcinogen 4-NQO. In the model, we observed dysplasia and tumors on the tongues of transgenic rats after treatment with 4-NQO in drinking water, however,

the incidence and multiplicity were greater in Tg rats than in non-Tg rats, and the onset of carcinogenesis was earlier in Tg rats. In addition, a series of pathological and immunohistochemical studies revealed that tongue squamous cell dysplasia, papilloma, carcinoma *in situ* and invasive carcinoma occurred in Tg rats. Five biomarkers (cyclin D1, GST-P, COX-2, iNOS and  $\beta$ -catenin) were expressed in these tongue squamous cell lesions and their expression increased with the disease progression. The changes of these markers were correlated with those of DNA ploidy patterns. Finally, we found that three reported cancer chemopreventive agents could inhibit the carcinogenesis in the Tg rat tongue caused by the treatment of 4-NQO. Taken together, we concluded that this Tg rat model could thus be used in the analysis and the chemoprevention/treatment of oral carcinogenesis.

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## Dietary administration with prenyloxycoumarins, auraptene and collinin, inhibits colitis-related colon carcinogenesis in mice

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We previously reported the chemopreventive ability of a prenyloxy-coumarin auraptene in chemically induced carcinogenesis in digestive tract, liver and urinary bladder of rodents. The current study was designed to determine whether dietary feeding of auraptene and its related prenyloxycoumarin collinin can inhibit colitis-related mouse colon carcinogenesis. The experimental diets, containing the compounds at 2 dose levels (0.01 and 0.05%), were fed for 17 weeks to male CD-1 (ICR) mice that were initiated with a single intraperitoneal injection of azoxymethane (AOM, 10 mg/kg body weight) and promoted by 1% (w/v) DSS in drinking water for 7 days. Their tumor inhibitory effects were assessed at week 20 by counting the incidence and multiplicity of colonic neoplasms and the immunohistochemical expression of proliferating cell nuclear antigen (PCNA)-labeling index, apoptotic index, cyclooxygenase (COX)-2, inducible nitric oxide (iNOS) and nitrotyrosine in colonic epithelial malignancy. Feeding with auraptene or collinin, at both doses, significantly inhibited the occurrence of colonic adenocarcinoma. In addition, feeding with auraptene or collinin significantly lowered the positive rates of PCNA, COX-2, iNOS and nitrotyrosine in adenocarcinomas, while the treatment increased the apoptotic index in colonic malignancies. Our findings may suggest that certain prenyloxycoumarins, such as auraptene and collinin, could serve as an effective agent against colitis-related colon cancer development in rodents.

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**Key words:** auraptene; azoxymethane; collinin; colitis-related carcinogenesis; dextran sodium sulfate

Colorectal cancer (CRC) is one of the most serious complications of inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease.<sup>1</sup> Long-term UC patients have high risk of developing CRC, when compared with the general population.<sup>2</sup> The precise mechanisms of the IBD-related carcinogenesis process are largely unclear, although it is generally assumed that chronic inflammation influences the development of IBD-related carcinogenesis.<sup>3</sup>

Fighting IBD-related CRC as well as sporadic CRC, by cancer chemoprevention strategy, is important to reduce the risk, and thus primary prevention of CRC in IBD has recently been receiving more attention. Previous experimental and epidemiological investigations suggest that several agents, such as folic acid,<sup>4</sup> conjugated linoleic acid,<sup>5</sup> ursodeoxycholic acid,<sup>6</sup> 5-aminosalicylic acid<sup>7</sup> and aspirin, may reduce the occurrence of CRC in patients with IBD.<sup>8,9</sup> Consistent with these data, several nonsteroidal anti-inflammatory drugs (NSAIDs), including cyclooxygenase (COX)-2 inhibitors, suppressed the development of chemically induced colon carcinomas in rats<sup>10</sup> and intestinal polyps in *Min* mice, with a nonsense mutation of the *Apc* gene.<sup>11</sup> In addition, clinical trials demonstrated that intake of a NSAID, sulindac, causes regression of adenomas in patients with familial adenomatous polyposis.<sup>12</sup>

Epidemiological studies indicate an inverse correlation between the intake of fruits/vegetables and human colon cancer.<sup>13</sup> Thus, primary prevention, including chemoprevention, using the active compounds in fruits and vegetables is also important for reducing the risk of this malignancy. Citrus fruit contains several chemopreventive compounds against colon cancer.<sup>14–17</sup> Prenyloxycoumarins, including auraptene (Fig. 1a) and collinin (Fig. 1b), are candi-

dates of such chemopreventers. They are secondary metabolites, mainly found in plants belonging to the families of Rutaceae and Umbelliferae. Several of these coumarins were shown to possess valuable pharmacological properties. These compounds were reported to have anti-inflammatory activity.<sup>18</sup> Auraptene significantly attenuated the lipopolysaccharide (LPS)-induced protein expression of inducible nitric oxide synthase (iNOS) and COX-2, with decreases in production of nitric anion and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), and yet suppressed the release of tumor necrosis factor (TNF)- $\alpha$  and I $\kappa$ B degradation.<sup>19,20</sup> Furthermore, auraptene and collinin also cause complete inhibition of platelet aggregation, induced by arachidonic acid and platelet activated factor *in vitro*.<sup>21</sup> We have previously found that a citrus auraptene suppresses chemically induced carcinogenesis in rodents.<sup>22–24</sup>

For understanding the pathogenesis of IBD and IBD-related CRC, several animal models have been established. Most used is a mouse model with dextran sodium sulfate (DSS).<sup>25</sup> Modifying effects of several xenobiotics on CRC-related colon carcinogenesis have been reported,<sup>26,27</sup> using this model. However, this colitis model using DSS, with or without carcinogen, needs to a long period repeated administration of DSS to induce colitis and colitis-related CRC that mimic human UC. To investigate the pathogenesis in IBD-related CRC and search novel and effective chemopreventive agents against this type of malignancy, we developed a novel colitis-related mouse CRC model, using a colon carcinogen azoxymethane (AOM) and DSS, in which large bowel adenocarcinomas develop within a short-term period, and their histology and biological alteration resemble to those found in humans.<sup>28</sup> Our animal model indicates that in the large bowel, inflammation induced by DSS strongly promotes the development of epithelial malignant neoplasia. Oxidative/nitrosative stress caused by DSS exposure may contribute the development of high incidence of colonic adenocarcinomas.<sup>29,30</sup> Recently, we demonstrated that dietary administration of COX-2 inhibitor and peroxisome proliferator-activated receptor ligands suppressed colitis-related colonic carcinogenesis, using our mouse colon carcinogenesis model.<sup>31</sup>

As a part of our search for safer chemopreventive agents against colitis-related colon cancer, we examined, in the present study, the

**Abbreviations:** AOM, azoxymethane; CRC, colorectal cancer; COX, cyclooxygenase; DSS, dextran sodium sulfate; FAP, familial adenomatous polyposis; H & E, hematoxylin and eosin; IBD, inflammatory bowel disease; iNOS, inducible nitric oxide synthase; NSAIDs, nonsteroidal anti-inflammatory drugs; PCNA, proliferating cell nuclear antigen; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; ssDNA, single stranded DNA; TNF, tumor necrosis factor; UC, ulcerative colitis.

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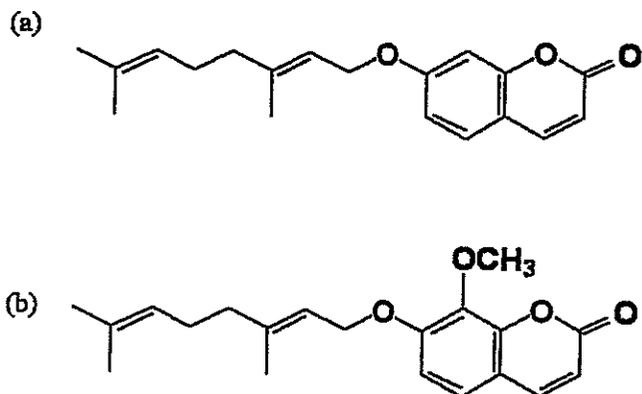


FIGURE 1 – Chemical structures of (a) auraptene and (b) collinin.

effects of auraptene and collinin on our mouse colon carcinogenesis model.<sup>28</sup>

### Material and methods

#### Animals, chemicals and diets

Male Crj: CD-1 (ICR) (Charles River Japan, Tokyo, Japan), aged 5 weeks, were used in this study. They were maintained at Kanazawa Medical University Animal Facility, according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (5 or 6 mice/cage), with free access to drinking water and a pelleted basal diet, CRF-1 (Oriental Yeast Co., Tokyo, Japan), under controlled conditions of humidity ( $50 \pm 10\%$ ), light (12/12 hr light/dark cycle) and temperature ( $23 \pm 2^\circ\text{C}$ ). They were quarantined for the first 7 days, and then randomized by body weight into experimental and control groups. A colonic carcinogen AOM was purchased from Sigma Chemical Co. (St. Louis, MO). DSS with a molecular weight of 36,000–50,000 was purchased from ICN Biochemicals (Aurora, OH). DSS for induction of colitis was dissolved in water at a concentration of 1% (w/v). Auraptene (99.6% purity)<sup>32</sup> and collinin (99.8% purity)<sup>18</sup> were synthesized, as described previously. Experimental diet, containing auraptene or collinin, was prepared every week by mixing the respective compound in powdered basal diet CRF-1, at a concentration (w/w) of 0.01 or 0.05%. The dose levels of the 2 compounds were selected on the basis of our previous experiments.<sup>18,22–24</sup>

#### Experimental procedures

A total of 75 male ICR mice were divided into 10 (experimental and control) groups (Fig. 2). Mice in groups 1 through 5 were given a single intraperitoneal injection of AOM (10 mg/kg body weight). Starting 1 week after the injection, animals were administered to 1% DSS in drinking water for 7 days, and then followed without any further treatment for 15 weeks. Mice of group 1 were maintained on basal diet, throughout the study. Mice in groups 2 through 5 were given 0.01% auraptene in diet (group 2), 0.05% auraptene in diet (group 3), 0.01% collinin in diet (group 4) or 0.05% collinin in diet (group 5), respectively, for 17 weeks, starting 1 week after the stop of DSS administration. Group 6 was given a single dose of AOM. Group 7 was given 1% DSS for 7 days. Animals in groups 8 and 9 were given the diets containing 0.05% auraptene and 0.05% collinin alone, respectively. Group 10 consisted of untreated mice. All animals were killed at the end of the study (week 20). Their large bowels were flushed with saline, excised, their length measured (from ileocecal junction to the anal verge) and cut open longitudinally along the main axis, and then washed with saline. The large bowels were macroscopically inspected, cut and fixed in 10% buffered formalin, for at least 24 hr. Histological examination was performed on paraffin-embedded sections, after

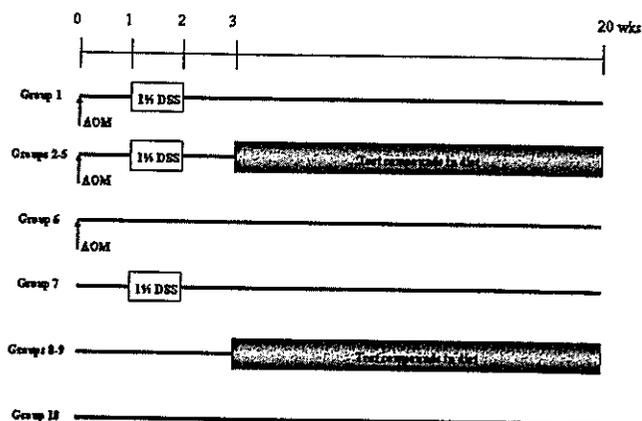


FIGURE 2 – Experimental protocol.

hematoxylin and eosin (H & E) staining. Colonic neoplasms were diagnosed, according to the description by Ward.<sup>33</sup> Grade of colitis was scored, and the sections were stained with H & E,<sup>29,34</sup> from all groups.

#### Immunohistochemistry

Immunohistochemistry for proliferating cell nuclear antigen (PCNA), apoptotic nuclei, COX-2, iNOS and nitrotyrosine was performed on 4- $\mu\text{m}$ -thick paraffin-embedded sections, from the colons of mice in each group 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  $65^\circ\text{C}$ , deparaffinized in xylene and rehydrated through graded ethanols at room temperature. A 0.05 M Tris HCl buffer (pH 7.6) was used to prepare solutions, and for washes between various steps. Incubations were performed in a humidified chamber. For the determination of PCNA-incorporated nuclei, the PCNA-immunohistochemistry was performed.<sup>35</sup> Apoptotic index was also evaluated by immunohistochemistry for single stranded DNA (ssDNA).<sup>35</sup> Sections were treated for 40 min at room temperature, with 2% BSA, and incubated overnight at  $4^\circ\text{C}$  with primary antibodies, such as anti-PCNA mouse monoclonal antibody (diluted 1:50; PC10, DAKO Japan), anti-ssDNA rabbit polyclonal antibody (diluted 1:300, DAKO Japan), anti-COX-2 rabbit polyclonal antibody (diluted 1:50, IBL Co., Gunma, Japan), anti-iNOS rabbit polyclonal antibody (diluted 1:1,000, Wako Pure Chemical Industries, Osaka, Japan), and anti-nitrotyrosine rabbit polyclonal antibody (diluted 1:500, Upstate Biotechnology, Lake Placid, NY). To reduce the nonspecific staining of mouse tissue by the mouse antibodies, a Mouse On Mouse IgG blocking reagent (Vector Laboratories, Burlingame, CA) was applied for 1 hr. Horseradish peroxidase activity was visualized by treatment with  $\text{H}_2\text{O}_2$  and 3,3'-diaminobenzidine 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. On the control sections, incubation with the primary antibodies was omitted.

Intensity and localization of immunoreactivities, against all primary antibodies used, were examined on all sections using a microscope (Olympus BX41, Olympus Optical Co., Tokyo, Japan). The PCNA and apoptotic indices were determined, by counting the number of positive cells among at least 200 cells in the lesion, and were indicated as percentages. Each slide for COX-2, iNOS and nitrotyrosine was evaluated for intensity of immunoreactivity on a 0 to 4+ scale. The overall intensity of the staining reaction was scored, with 0 indicating no immunoreactivity and no positive cells, 1+ weak immunoreactivity and <10% of positive cells, 2+ mild immunoreactivity and 10–30% of positive cells, 3+ moderate

immunoreactivity and 31–60% of positive cells, and 4+ strong immunoreactivity and 61–100% of positive cells.

#### Statistical analysis

Measurements were compared by Bonferroni multiple comparison post test or Fisher's exact probability test. Differences were considered statistically significant at  $p < 0.05$ .

### Results

#### General observation

Bloody stool was observed in a few mice, which received 1% DSS, and their body weight gains were slightly decreased during the period of treatment. However, thereafter no such clinical symptoms were noted. Body weights, liver weights and relative liver weights, in all groups at the end of the study, are shown in Table I. The mean body weights, liver weights and relative liver weights did not significantly differ among the groups. The mean length of large bowel in groups 2–5 was lower than group 1, but the differences did not reach statistical significance. Histologically, there were no pathological alterations suggesting toxicity of auroptene and collinin in the liver, kidneys, lung and heart. Food consumption (g/day/mice) did not significantly differ among the groups (data not shown).

#### Pathological findings

Macroscopically, nodular or polypoid colonic tumors were observed in the middle and distal colon of mice in groups 1 through 5. Histopathologically, AOM/DSS treated mice showed dysplasia (Fig. 3a), adenoma (Fig. 3b) and adenocarcinoma (Fig. 3c). These tumors histologically diagnosed as tubular adenoma or well-/moderately-differentiated tubular adenocarcinoma. Animals of groups 6–10 did not have large bowel neoplasms in any organs examined, including the colon. The incidences and multiplicity of colon neoplasms are shown in Table II, respectively. Group 1 (AOM/DSS) induced 100% incidence of colon adenocarcinomas, with a multiplicity of  $3.00 \pm 1.41$ . The incidences of colorectal adenocarcinomas in groups 2 (AOM/DSS/0.01% auroptene), 3 (AOM/DSS/0.05% auroptene), 4 (AOM/DSS/0.01% collinin) and 5 (AOM/DSS/0.05% collinin) were significantly smaller than that of group 1 ( $p < 0.02$ ,  $p < 0.01$ ,  $p < 0.01$  and  $p < 0.01$ , respectively). The mul-

tiplicity of colon adenocarcinomas in groups 2, 3, 4 and 5 were also significantly lower than that of group 1 ( $p < 0.005$ ,  $p < 0.001$ ,  $p < 0.005$  and  $p < 0.001$ , respectively). Colitis was present with or without colonic dysplasia in the middle or distal colon of mice treated with DSS. As shown in Fig. 4, colonic inflammation scores in groups 3 ( $p < 0.05$ ) and 5 ( $p < 0.05$ ) were significantly decreased, when compared with that in group 1.

#### Immunohistochemistry for PCNA, ssDNA, COX-2, iNOS and nitrotyrosine in colonic adenocarcinoma

As summarized in Table III, PCNA-labeling index of colonic adenocarcinomas developed in groups 2 ( $p < 0.01$ ), 3 ( $p < 0.01$ ), 4 ( $p < 0.01$ ) and 5 ( $p < 0.05$ ) was significantly smaller than group 1 (Figs. 5a–5c), and apoptotic index, measured by ssDNA immunohistochemistry in groups 2 ( $p < 0.05$ ), 4 ( $p < 0.05$ ) and 5 ( $p < 0.01$ ), was significantly greater than group 1 (Figs. 5d–5f). Scores for COX-2 and iNOS expression in colonic adenocarcinomas is also given in Table III. In the positive cases of COX-2 and iNOS expression in the dysplasia and adenocarcinoma, the staining pattern was granular and localized to cytoplasm or nuclei or both. Slight immunoreactivity for COX-2 and iNOS was observed in the superficial layers of the nonlesional colonic mucosa and in parts of basal layer, in all groups. COX-2 expression scores of colonic adenocarcinomas in groups 2 ( $p < 0.01$ ), 3 ( $p < 0.05$ ) and 5 ( $p < 0.05$ ) and that of iNOS in groups 2 ( $p < 0.001$ ), 3 ( $p < 0.001$ ), 4 ( $p < 0.01$ ) and 5 ( $p < 0.01$ ) were significantly decreased, when compared with that in group 1 (Figs. 5g–5i for COX-2 and Figs. 5j–5l for iNOS). Nitrotyrosine immunoreactivity (Figs. 5m–5o) was mainly observed in mononuclear cells infiltrated in the colonic mucosa with the lesions, and the stainability was relatively weak in the neoplastic cells. The score of nitrotyrosine is also given in Table III. The scores of groups 3 ( $p < 0.05$ ) and 5 ( $p < 0.05$ ) were significantly higher than that of group 1. The scores of groups 2 and 4 were also lower than that of group 1, but the differences were insignificant.

### Discussion

The results of the present work clearly indicated that 2 prenyloxycoumarins, auroptene and collinin, effectively inhibited AOM/DSS-induced colitis-related colonic carcinogenesis, without any adverse effects in mice. The suppressive effect of auroptene

TABLE I – BODY, LIVER, RELATIVE LIVER WEIGHTS AND LENGTH OF LARGE BOWEL

Group no.	Treatment	Body wt (g)	Liver wt (g)	Relative liver wt (g/100 g body wt)	Length of colon (cm)
1	AOM + 1%DSS (10) <sup>1</sup>	42.3 ± 2.4 <sup>2</sup>	2.5 ± 0.5	5.90 ± 0.96	14.6 ± 1.1
2	AOM + 1%DSS/0.01% auroptene (10)	42.7 ± 2.4	2.6 ± 0.2	6.13 ± 0.55	15.2 ± 0.9
3	AOM + 1%DSS/0.05% auroptene (10)	42.4 ± 3.1	2.7 ± 0.3	6.45 ± 0.56	15.2 ± 0.9
4	AOM + 1%DSS/0.01% collinin (10)	48.1 ± 7.6	2.9 ± 0.6	5.96 ± 0.54	15.0 ± 1.1
5	AOM + 1%DSS/0.05% collinin (10)	45.5 ± 5.7	2.4 ± 0.3	5.20 ± 0.36	15.2 ± 1.2
6	AOM alone (5)	47.9 ± 6.8	3.0 ± 0.5	6.31 ± 0.26	16.5 ± 0.3
7	1% DSS alone (5)	44.6 ± 3.2	2.8 ± 0.3	6.32 ± 0.54	14.9 ± 1.0
8	0.05% auroptene (5)	47.0 ± 5.5	2.6 ± 0.2	5.64 ± 0.38	16.5 ± 0.7
9	0.05% collinin (5)	44.4 ± 3.1	2.8 ± 0.3	6.25 ± 0.64	15.7 ± 0.9
10	None (5)	44.0 ± 2.6	2.8 ± 0.4	6.36 ± 0.61	16.5 ± 1.0

<sup>1</sup>Values in parentheses indicate the numbers of mice examined. –<sup>2</sup>Mean ± SD.



FIGURE 3 – Histopathology of colonic lesions. (a) Dyplastic crypts, (b) tubular adenoma and (c) tubular adenocarcinoma developed in a mouse from group 1. H & E stain, original magnification, (a) ×20, (b, c) ×4.

TABLE II - INCIDENCE AND MULTIPLICITY OF COLONIC NEOPLASIA

Group no.	Treatment	No. of mice	Incidence (no. of mice with neoplasms)			Multiplicity (no. of tumors/mice)		
			Total	AD <sup>1</sup>	ADC <sup>2</sup>	Total	AD	ADC
1	AOM + 1% DSS	10	10/10 (100) <sup>3</sup>	10/10 (100)	10/10 (100)	5.40 ± 1.71 <sup>4</sup>	2.40 ± 1.07	3.00 ± 1.41
2	AOM + 1% DSS/0.01% auraptene	10	8/10 (80)	8/10 (80)	5/10 (50) <sup>5</sup>	3.10 ± 2.28	2.10 ± 1.79	1.00 ± 1.33 <sup>6</sup>
3	AOM + 1% DSS/0.05% auraptene	10	6/10 (60) <sup>7</sup>	6/10 (60) <sup>7</sup>	4/10 (40) <sup>8</sup>	1.70 ± 1.70 <sup>9</sup>	1.10 ± 1.29	0.60 ± 0.84 <sup>9</sup>
4	AOM + 1% DSS/0.01% collinin	10	7/10 (70)	6/10 (60) <sup>7</sup>	4/10 (40) <sup>8</sup>	2.90 ± 2.33	2.00 ± 1.83	0.90 ± 1.20 <sup>6</sup>
5	AOM + 1% DSS/0.05% collinin	5	6/10 (60) <sup>7</sup>	5/10 (50) <sup>5</sup>	4/10 (40) <sup>8</sup>	1.40 ± 1.43 <sup>9</sup>	0.80 ± 0.92	0.60 ± 0.84 <sup>9</sup>
6	AOM alone	5	0/5 (0)	0/5 (0)	0/5 (0)	0	0	0
7	1% DSS alone	5	0/5 (0)	0/5 (0)	0/5 (0)	0	0	0
8	0.05% auraptene	5	0/5 (0)	0/5 (0)	0/5 (0)	0	0	0
9	0.05% collinin	5	0/5 (0)	0/5 (0)	0/5 (0)	0	0	0
10	None	5	0/5 (0)	0/5 (0)	0/5 (0)	0	0	0

<sup>1</sup>AD, adenoma. <sup>2</sup>ADC, adenocarcinoma. <sup>3</sup>Values in parentheses indicate percentages. <sup>4</sup>Mean ± SD. <sup>5-9</sup>Significantly different from group 1 by Fisher's exact probability test or Bonferroni multiple comparison post test. (<sup>5</sup> $p < 0.02$ , <sup>6</sup> $p < 0.005$ , <sup>7</sup> $p < 0.05$ , <sup>8</sup> $p < 0.01$ , and <sup>9</sup> $p < 0.001$ ).

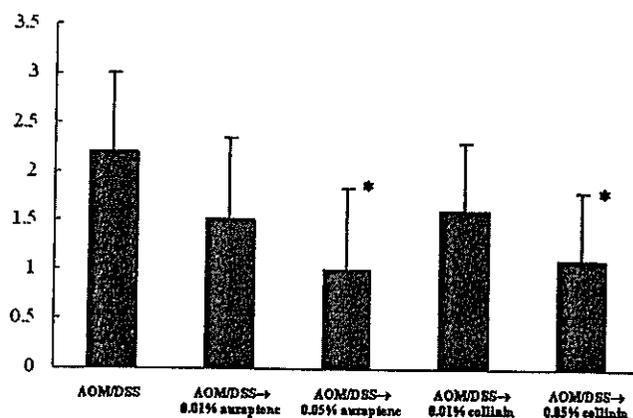


FIGURE 4 - Inflammation score. Statistical analysis using Bonferroni multiple comparison post test indicates significant difference (\* $p < 0.05$ ), vs. the AOM/DSS group.

and collinin on the development of colonic adenocarcinoma was well correlated with the inhibition of cell proliferation activity, induction of apoptosis and inhibition of immunoreactivity of COX-2 and iNOS in the colonic malignancies. These findings may suggest that dietary auraptene and collinin suppress IBD-associated colon carcinogenesis and are possibly applicable in human clinical trials.

The pathogenesis of IBD-associated colorectal carcinogenesis is widely believed to involve a stepwise progression from inflamed and hyperplastic cryptal cells, through flat dysplasia, to finally adenocarcinoma,<sup>36</sup> but the mechanism is still unclear. However, mucosal inflammation may result in colonic carcinogenesis through several proposed mechanisms, such as induction of genetic mutations, increased-cryptal cell proliferation, changes in crypt cell metabolism and bile acid enterohepatic circulation, and alterations in bacteria flora.<sup>37,38</sup> These events are considered to promote IBD-associated CRC development. In the colon, the number of epithelial cells in the crypts is strictly regulated by a balance between cell proliferation and cell death that maintains homeostasis.<sup>39</sup> In neoplastic tissues, changes in cell proliferation and apoptosis are regarded as a common denominator in the pathogenesis of tumor formation.<sup>40</sup> It is thought that intermittent colonic epithelial damage and restitution caused by chronic inflammation contribute to the increased cancer risk in the long-term UC patients. The elevated rate of cell turnover associated with the epithelial damage-restitution cycle may increase the occurrence of mitotic aberrations and other genetic and epigenetic changes, as well as take part in the pro-

motion stage of cancer development.<sup>41</sup> In the present study, the modifying effects of auraptene and collinin on the cellular proliferation and apoptosis may contribute to their lowering activity in the incidence and multiplicity of colon adenocarcinomas.

Chronic inflammation is recognized as one of the major causes of human cancer.<sup>42,43</sup> Inflammation-caused oxidative/nitrosative cellular damage is suspected to be responsible for the development of IBD-associated colorectal neoplasms. Therefore, certain antioxidants are effective as cancer chemopreventive agents. Auraptene suppresses 12-*O*-tetradecanoylphorbol-13-acetate-induced superoxide in HL-60 cells, attenuates inflammatory leukocyte activation *in vivo*, and decreases inflammation, H<sub>2</sub>O<sub>2</sub> production and cell proliferation.<sup>44</sup> In addition, auraptene quite likely reduces the production of lipid peroxidation products in rat colon carcinogenesis.<sup>24</sup> These findings suggest that auraptene mitigates oxidative stress by suppressing oxygen radical generation by inflammatory leukocytes. Since nitrotyrosine production may involve in CRC development in this colitis-related mouse colon carcinogenesis model,<sup>29,30</sup> our results suggesting potential use of the antioxidants, collinin and auraptene, in the prevention of IBD-associated cancer may be caused by their suppression of oxidative/nitrosative cellular damage in our model.

There are an increasing number of reports showing that the expression of COX-2 and iNOS is closely associated with the development of cancers.<sup>45,46</sup> We also observed increased expression of COX-2 and iNOS in colon adenocarcinomas in this animal model.<sup>28</sup> The increases in the reaction products of iNOS and COX-2, nitric oxide and PGE<sub>2</sub> respectively, could contribute to colon tumorigenesis. Expression and activity of iNOS are increased in the colonic mucosa in patients with IBD<sup>47</sup> and colonic adenomas.<sup>48</sup> Several studies, using experimental colon carcinogenesis models, indicate that chemically induced colon tumors have higher expression or activity of iNOS or both, when compared with those found in the adjacent colonic tissue.<sup>26,49</sup> An iNOS-selective inhibitor could suppress the development of AOM-induced colonic preneoplastic lesions by inhibition of iNOS activity.<sup>50</sup> Likewise, an increased COX-2 expression is reported in human and rodent CRC,<sup>51,52</sup> and its overexpression may confer a survival advantage on cells by inhibition apoptosis and a change in cellular adhesion to the extracellular matrix.<sup>53</sup> Given the correlation between increased COX-2 expression and cancer occurrence in the inflamed colon, the chemopreventive effect of NSAIDs seems to be mediated, at least in part, by COX inhibition.<sup>54</sup> Our previous study<sup>55</sup> and those of others<sup>56,57</sup> shows that COX-2 inhibitors inhibited colon tumorigenesis as well as colitis, induced by naturally occurring carcinogen. Suh *et al.*<sup>58</sup> synthesized novel synthetic triterpenoids that suppressed iNOS and COX-2 protein expression, and demonstrated their potent differentiating, antiproliferating and anti-inflammatory activities.<sup>59</sup> Auraptene also can

TABLE III - PCNA AND APOPTOSIS INDICES AND SCORES OF COX-2, iNOS AND NITROTYROSINE EXPRESSION IN COLONIC ADENOCARCINOMAS

Group no.	Treatment (no. of mice examined)	PCNA-labeling index (%)	Apoptotic index (%)	COX-2	iNOS	Nitrotyrosine
1	AOM + 1% DSS	68.2 ± 10.5 <sup>1</sup> (20) <sup>2</sup>	11.4 ± 5.8(20)	3.6 ± 0.6(20)	3.7 ± 0.5(20)	2.5 ± 0.8(20)
2	AOM + 1% DSS/0.01% auraptene	50.0 ± 12.6 <sup>3</sup> (10)	18.1 ± 5.0 <sup>4</sup> (10)	2.4 ± 1.2 <sup>5</sup> (10)	2.3 ± 0.8 <sup>5</sup> (10)	1.7 ± 0.8(10)
3	AOM + 1% DSS/0.05% auraptene	47.2 ± 13.4 <sup>3</sup> (6)	20.7 ± 5.4(6)	2.0 ± 0.9 <sup>5</sup> (6)	1.8 ± 1.0 <sup>5</sup> (6)	1.4 ± 0.7 <sup>5</sup> (6)
4	AOM + 1% DSS/0.01% collinin	51.8 ± 10.0 <sup>3</sup> (9)	19.1 ± 5.6 <sup>4</sup> (9)	2.6 ± 1.0(9)	2.4 ± 0.7 <sup>5</sup> (9)	1.8 ± 0.8(9)
5	AOM + 1% DSS/0.05% collinin	49.3 ± 13.2 <sup>4</sup> (6)	21.3 ± 6.9 <sup>5</sup> (6)	2.3 ± 1.2 <sup>5</sup> (6)	2.2 ± 1.3 <sup>5</sup> (6)	1.3 ± 0.5 <sup>4</sup> (6)

<sup>1</sup>Mean ± SD. <sup>2</sup>Numbers in parentheses are the numbers of lesions examined. <sup>3-5</sup>Significantly different from group 1 by Bonferroni multiple comparison post test. (<sup>3</sup> $p < 0.01$ , <sup>4</sup> $p < 0.05$  and <sup>5</sup> $p < 0.001$ ).

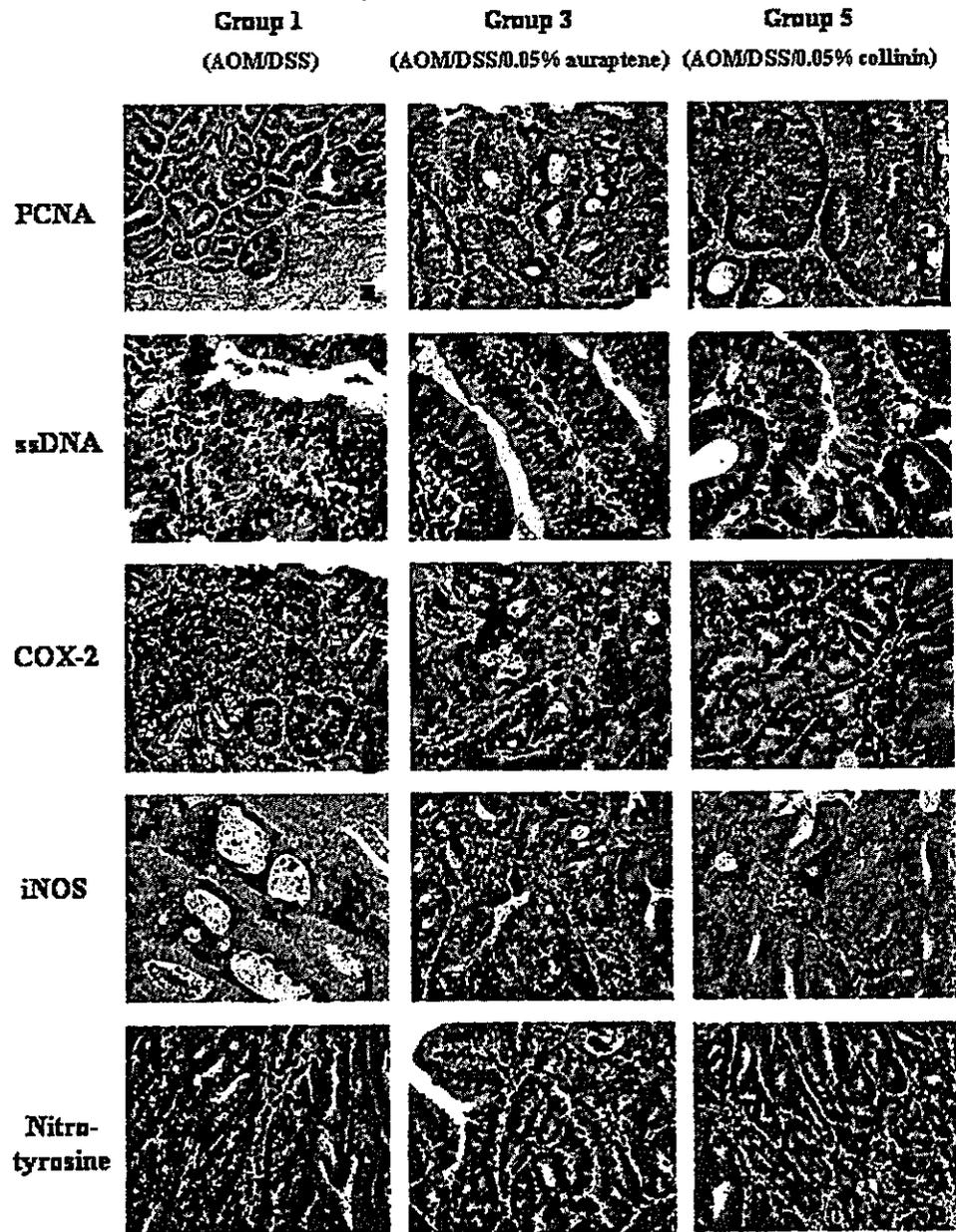


FIGURE 5 - Immunohistochemistry of PCNA, ssDNA, COX-2, iNOS and nitrotyrosine in adenocarcinomas. When compared to group 1 (a), the numbers of PCNA-positive nuclei in adenocarcinomas developed in mice from groups 3 (b) and 5 (c) were low. In contrast to ssDNA positivity (d) in adenocarcinoma cell nuclei (group 1), only a few positive nuclei were found in adenocarcinoma cells in groups 3 (e) and 5 (f). Stainability of COX-2 (g), iNOS (j) and nitrotyrosine (m) immunohistochemistry of adenocarcinoma cells developed in a mouse from group 1 was strong, but the immunohistochemical reaction for COX-2 in groups 3 (h) and 5 (i), that for iNOS in groups 3 (k) and 5 (l), and that for nitrotyrosine in groups 3 (m) and 5 (o) were weak. (a-c) PCNA immunohistochemistry, (d-f) ssDNA immunohistochemistry, (g-i) COX-2 immunohistochemistry, (j-l) iNOS immunohistochemistry and (m-o) nitrotyrosine immunohistochemistry. Original magnification, (a, g) ×10, (b, c, h-o) ×20 and (d-f) ×40.

inhibit iNOS and COX-2 expression in RAW 264.7 cells treated with LPS and TNF- $\alpha$ .<sup>19</sup> Our recent study<sup>29</sup> indicated that changes of inflammation scores paralleled with those of the nitrotyrosine immunohistochemical scores in the colonic mucosa, and these alterations in the inflamed colon resulted in powerful promotion effect of DSS in the AOM/DSS-induced mouse colon carcinogene-

sis. In the current study, suppressing effects of dietary feeding with auraptene and collinin after treatment with AOM and DSS might be mainly due to their inhibition of inflammation and oxidative/nitrosative stress in the colon.

In conclusion, dietary administration with prenyloxycoumarins, auraptene and collinin, could effectively suppress colitis-related

colon carcinogenesis, induced by AOM and DSS in male ICR mice. Our on-going study on molecular profiles in colonic samples from the current experiment will provide precise molecular mechanisms involved in their inhibitory action in AOM/DSS-induced mouse colon carcinogenesis.

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## Preventive effects of chrysin on the development of azoxymethane-induced colonic aberrant crypt foci in rats

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**Abstract.** The modifying effects of dietary feeding with chrysin (5,7-dihydroxyflavone) on the development of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) were investigated in male F344 rats. We also assessed the effect of chrysin on mitosis and apoptosis in 'normal appearing' crypts. To induce ACF, rats were given two weekly subcutaneous injections of AOM (20 mg/kg body weight). They also received an experimental diet containing chrysin (0.001 or 0.01%) for 4 weeks, starting 1 week before the first dose of AOM. AOM exposure produced a substantial number of ACF (73±13/rat) at the end of the study (week 4). Dietary administration of chrysin caused significant reduction in the frequency of ACF: 0.001% chrysin, 37±17/rat (49% reduction, P<0.001); and 0.01% chrysin, 40±10/rat (45% reduction, P<0.001). In addition, chrysin administration significantly reduced the mitotic index and significantly increased the apoptotic index in 'normal appearing' crypts. These findings might suggest a possible chemopreventive activity of chrysin in the early step of colon tumorigenesis through modulation of cryptal cell proliferation activity and apoptosis.

### Introduction

Colorectal cancer is one of the leading causes of cancer death in Western countries. Globally, colorectal cancer accounted for approximately 1 million new cases in 2002 (9.4% of the world) and mortality is approximately one half that of incidence (~529,000 deaths in 2002) (1). In Japan, its incidence has been increasing and colonic malignancy is now the third leading cause of cancer death. In this context, primary prevention,

including chemoprevention, is important for fighting this malignancy.

Flavonoids are plant secondary metabolites ubiquitously distributed throughout the plant kingdom, and numerous reports have shown their biological effects, such as anti-oxidative and anti-inflammatory activity. They also act as inhibitors of several enzymes that are activated in certain inflammatory conditions (2), while a variety of cell types associated with the immune system are down-regulated by certain flavonoids *in vitro* (3). Further, most flavonoids show potent anti-oxidative/radical scavenging effects (4). A natural flavonoid, chrysin (5,7-dihydroxyflavone, Fig. 1), which is a potent inhibitor of the enzyme, CYP1A (5), and aromatase (6), is present in many plants, honey, and propolis (7,8). Studies have shown that chrysin suppresses lipopolysaccharide (LPS)-induced cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) expression through the activation of peroxisome proliferator-activated receptor (PPAR)- $\gamma$  (9). In our previous studies, a polymethoxy flavonoid, nobiletin (5,6,7,8,3',4'-hexamethoxyflavone), suppressed the expression of proinflammatory genes, such as iNOS and COX-2, *in vitro* (10) and inhibited azoxymethane (AOM)-induced rat colon carcinogenesis (11). In addition, pomegranate (*Punica granatum L.*) seed oil, which contains more than 70% conjugated linolenic acids, in the diet suppressed AOM-induced colon carcinogenesis in rats through an up-regulation of PPAR $\gamma$  protein in the non-tumorous colonic mucosa (12). Thus, proinflammatory genes and PPAR $\gamma$  are good targets for chemoprevention of colon carcinogenesis.

Recently, several *in vitro* studies have shown that chrysin is able to inhibit the growth of HeLa cells by downregulating the expression of proliferating cell nuclear antigen (PCNA) (13), induce apoptosis via caspase activation and Akt inactivation in U937 leukemia cells (14), and cause cell-cycle arrest in human colon cancer cells (15), and C6 glioma cells (16). However, there are few reports investigating whether chrysin has cancer chemopreventive effects on the colon in experimental animal studies.

In the current study, we investigated the possible suppressing effect of chrysin on the occurrence of AOM-induced aberrant crypt foci (ACF), which are putative preneoplastic lesions for colonic adenocarcinoma (17-19), with a short-term rat ACF bioassay. In addition, we assess

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**Key words:** chrysin, aberrant crypt foci, mitosis, apoptosis

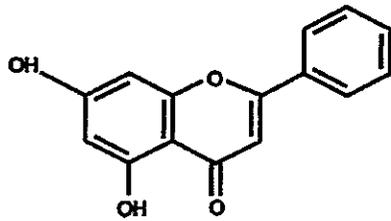


Figure 1. Chemical structure of chrysin.

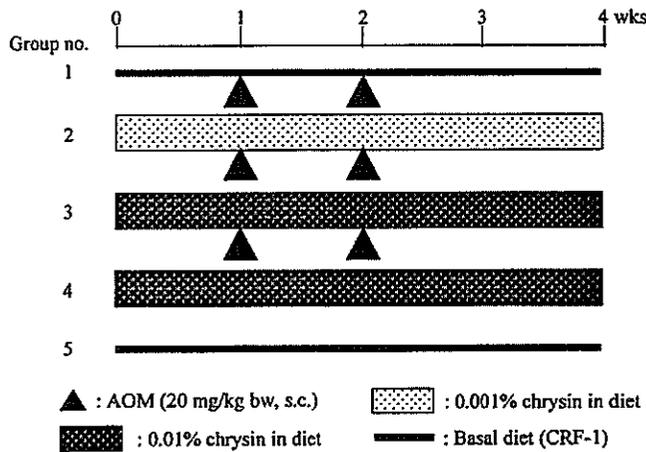


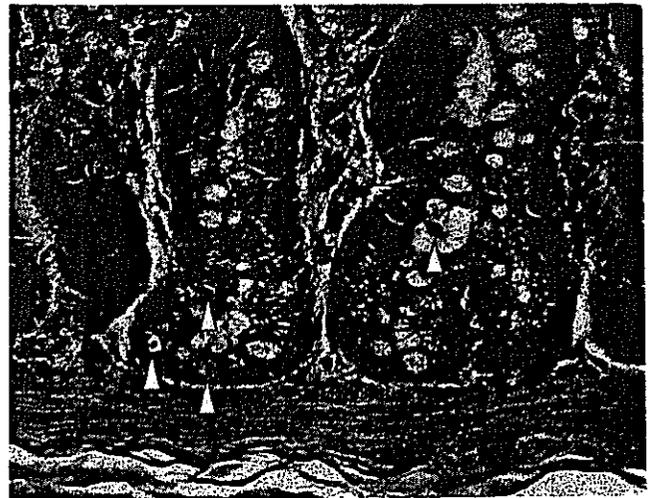
Figure 2. Experimental protocol.

whether dietary chrysin affects cell proliferation activity and induces apoptosis in the colonic epithelium, since certain chemopreventive agents exert cancer inhibitory action through reduction of cell proliferating activity (20) and induction of apoptosis (21) in the target tissue.

### Materials and methods

**Animals, chemicals and diet.** Male F344 rats (Charles River Japan, Inc, Kanazawa, Japan), aged 4 weeks, were used for an ACF assay. The animals were maintained in Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (4 rats/cage) with free access to tap water and a basal MF diet (Oriental Yeast, Co., Ltd., Nagoya, Japan) under controlled conditions of humidity ( $50 \pm 10\%$ ), lighting (12-h light/dark cycle), and temperature ( $23 \pm 2^\circ\text{C}$ ). They were quarantined for 7 days after arrival, and randomized by body weight into experimental and control groups. AOM for ACF induction was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Chrysin was obtained from Funakoshi Co. (Tokyo, Japan).

**Experimental procedure for ACF.** A total of 32 male F344 rats were divided into five experimental and control groups (Fig. 2). Animals in groups 1 through 3 were initiated with AOM by two weekly subcutaneous injections (20 mg/kg body weight) to induce colonic ACF. Rats in groups 2 and 3 were fed diets containing 0.001% and 0.01% chrysin for 4 weeks, respectively, starting one week before the first dose of



△ : apoptotic cells      ▲ : mitotic cell

Figure 3. Apoptotic and mitotic cells in the crypt from the distal colon, which was stained with hematoxylin and eosin, from a rat in group 3 (AOM+0.01% chrysin). Apoptotic cells are identified by cell shrinkage, homogeneous basophilic and condensed nuclei, nuclear fragments (apoptotic bodies), marked eosinophilic condensation of cytoplasm and sharply delineated cell borders surrounded by a clear halo. Yellow arrowheads indicate apoptotic cells and the black arrowhead indicates a mitotic cell.

AOM. Group 4 did not receive AOM and were given the diet containing 0.01% chrysin. Group 5 served as an untreated control. At week 4, rats were sacrificed under ether anesthesia to assess the occurrence of colonic ACF and we performed a careful necropsy, with emphasis on the colon, liver, kidney, lung, and heart. All grossly abnormal lesions in any tissue and the organs, e.g. liver (caudate lobe), kidney, lung, and heart, were fixed in 10% buffered formalin solution for histopathology.

**Determination of ACF.** The frequency of ACF was determined according to the method described in our previous report (22). At necropsy, the colons were flushed with saline, excised, 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. The fixed colons were dipped in a 0.5% solution of methylene blue in distilled water for 30 sec, and placed on a microscope slide to count the ACF.

**Counting mitotic and apoptotic cells.** To identify intramucosal apoptotic and mitotic cells in the crypts, the distal colon (2 cm from the anus) was cut out, embedded in paraffin, and 4  $\mu\text{m}$ -thick serial sections were made. The paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and evaluated under a light microscope for apoptotic and mitotic cells at a magnification of 400 (Fig. 3). 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 by a clear halo (23). The apoptotic and mitotic indices in the colonic crypts were determined on longitudinal sections that allowed evaluation of the whole crypt from the top to the base.

Table I. Body, liver, and relative liver weights.

Group no.	Treatment (no. of rats examined)	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
1	AOM alone (8)	194±8 <sup>a</sup>	9.7±0.7	5.00±0.68
2	AOM+0.001% chrysin (8)	192±7	10.5±1.1	5.47±0.45
3	AOM+0.01% chrysin (8)	195±5	9.9±0.5	5.10±0.18
4	0.01% chrysin (4)	203±7	10.5±0.9	5.14±0.28
5	No treatment (4)	196±9	9.4±0.5	4.80±0.17

<sup>a</sup>Mean ± SD.

Table II. Effect of chrysin on AOM-induced ACF formation in male F344 rats.

Group no.	Treatment (no. of rats examined)	Incidence (%)	Total no. of ACF/colon	Total no. of aberrant crypts/colon	No. of aberrant crypts/focus
1	AOM alone (8)	8/8 (100%)	73±13 <sup>a</sup>	145±28	1.98±0.10
2	AOM+0.001% chrysin (8)	8/8 (100%)	37±17 <sup>b</sup>	67±29 <sup>b</sup>	1.81±0.14 <sup>c</sup>
3	AOM+0.01% chrysin (8)	8/8 (100%)	40±10 <sup>b</sup>	69±21 <sup>b</sup>	1.73±0.09 <sup>b</sup>
4	0.01% chrysin (4)	0/4 (0%)	0	0	0
5	No treatment (4)	0/4 (0%)	0	0	0

<sup>a</sup>Mean ± SD, <sup>b</sup>significantly different from group 1 by one-way ANOVA with Bonferroni correction ( $P<0.001$ ), <sup>c</sup>significantly different from group 1 by one-way ANOVA with Bonferroni correction ( $P<0.05$ ).

Table III. Effect of chrysin on size of ACF induced by AOM.

Group no.	Treatment (no. of rats examined)	% of ACF containing:			
		1 crypt	2 crypts	3 crypts	≥ 4 crypts
1	AOM alone (8)	43.1±4.7 <sup>a</sup>	27.9±5.9	19.1±5.2	9.9±2.7
2	AOM+0.001% chrysin (8)	44.2±3.8	38.4±4.7 <sup>b</sup>	0.3±4.1 <sup>b</sup>	7.1±6.1
3	AOM+0.01% chrysin (8)	47.3±5.5	34.4±6.4	16.3±3.9 <sup>c</sup>	2.0±3.1 <sup>b</sup>

<sup>a</sup>Mean ± SD, <sup>b</sup>significantly different from group 1 by one-way ANOVA with Bonferroni correction ( $P<0.01$ ), <sup>c</sup>significantly different from group 2 by one-way ANOVA with Bonferroni correction ( $P<0.05$ ).

Randomly chosen crypts (28-57 crypts/colon) with well-oriented crypt structure from the mouth to the base were evaluated for counting apoptosis and mitosis. The apoptotic and mitotic indices were determined by dividing the total number of apoptotic or mitotic cells by the number of epithelial cells evaluated.

**Statistical evaluation.** Where applicable, data were analyzed using one-way ANOVA with Bonferroni correction (GraphPad Instat version 3.05, GraphPad Software, San Diego, CA, USA) with  $P<0.05$  as the criterion of significance.

## Results

**General observation.** All animals remained healthy throughout the experimental period. Food consumption (g/day/rat) did

not differ significantly among the groups (data not shown). As shown in Table I, the mean body, liver and relative liver weights (g/100 g body weight) in all groups did not differ significantly at the end of the study. Further, no significant pathological alternations were found in organs other than the colon.

**Frequency of ACF.** Table II summarizes the data on colonic ACF formation. All rats belonging to groups 1 through 3, which were treated with AOM, developed ACF. In groups 4 and 5, there was no microscopically observable change, including ACF, in colonic morphology. The mean number of ACF/colon in group 1 was 73±13. Dietary administration of chrysin (groups 2 and 3) significantly reduced the ACF incidence when compared to group 1: 49% reduction by 0.001% chrysin (group 2),  $P<0.001$ ; and 45% reduction by

Table IV. Epithelial proliferative kinetics in the distal colon.

Group no.	Treatment (no. of crypts examined)	Mitotic index (%)	Apoptotic index (%)	Crypt column height
1	AOM alone (44)	4.3±2.5 <sup>a,b</sup>	1.2±1.6	44.2±10.1 <sup>b</sup>
2	AOM+0.001% chrysin (38)	3.2±2.5	3.2±2.3 <sup>c</sup>	43.3±6.9
3	AOM+0.01% chrysin (57)	1.4±1.4 <sup>c</sup>	3.7±2.1 <sup>c</sup>	55.4±10.2 <sup>c</sup>
4	0.01% chrysin (56)	1.8±1.4	1.2±1.2	54.0±11.1 <sup>d</sup>
5	No treatment (28)	1.3±1.4	0.8±1.0	62.0±11.7

<sup>a</sup>Mean ± SD, <sup>b</sup>significantly different from group 5 by one-way ANOVA with Bonferroni correction (P<0.001), <sup>c</sup>significantly different from group 1 by one-way ANOVA with Bonferroni correction (P<0.001), <sup>d</sup>significantly different from group 5 by one-way ANOVA with Bonferroni correction (P<0.01)

0.01% chrysin (group 3), P<0.001. In addition, there were significant decreases in the total number of aberrant crypts (ACs) per colon (P<0.001), and in the number of ACs per focus in group 2 (9% reduction, P<0.05) and group 3 (13% reduction, P<0.001) when compared to group 1. The size distribution of ACF induced by AOM in groups 1-3 showed in Table III. The percentages of ACF consisting of one crypt did not significantly differ among these three groups. Although the percentage of ACF with 2 crypts in group 2 was significantly greater than that in group 1 (P<0.01), the values of ACF with 3 crypts in groups 2 and 3 were significantly smaller than in group 1 (P<0.01 and P<0.05, respectively). As for the percentage of ACF with ≥ 4 crypts, the value in group 3 was significantly lower than that in group 1 (P<0.01).

*Indices of mitosis and apoptosis in colonic crypts.* The data on the epithelial proliferative kinetics in 'normal appearing' distal colon are summarized in Table IV. The mitotic index was significantly higher in group 1 (4.3±2.5, 331% increase, P<0.001) than in group 5. The dietary administration of chrysin (groups 2 and 3) reduced the mitotic index in a dose-dependent manner when compared to group 1: 26% reduction by 0.001% chrysin (group 2); and 67% reduction by 0.01% chrysin, P<0.001 (group 3). Feeding with 0.01% chrysin alone (group 4) did not affect the mitotic index in the crypts. The apoptotic indices of groups 1, 4 and 5 were comparable, but the values in groups 2 and 3 were significantly increased when compared to group 1 (P<0.001). As for the crypt column height (no. of cells/crypt), the value in group 1 was significantly smaller than in group 5 (P<0.001). The crypt column height of group 3 was significantly larger than that of group 1 (P<0.001). The value in group 4 was significantly lower than in group 5 (P<0.01).

## Discussion

The results described here clearly indicate that dietary administration of chrysin at dose levels of 0.001% and 0.01% significantly inhibited AOM-induced ACF formation in male F344 rats. Moreover, the percentage of ACF that consisted of 4 or more aberrant crypts was significantly reduced by feeding with the diet supplemented with 0.01% chrysin. These findings indicate that dietary chrysin effectively suppresses the early phase of chemically-induced rat colon tumorigenesis. Also,

the inhibitory effect of chrysin (0.001%) in the diet on the formation of large ACF may suggest suppression of the late stage of AOM-induced colon carcinogenesis, since the number of large ACF is well correlated with the incidence of colonic adenocarcinoma induced by a colonic carcinogen, AOM (18,19,24). Our results are the first to show the chemopreventive ability of chrysin in ACF formation in an *in vivo* study with a colon carcinogenesis model.

The oral disposition of the dietary flavonoid, chrysin, in humans has been reported (25). Seven healthy subjects were administered 400 mg chrysin orally and the areas under the plasma concentration-time curves (AUCs) and urinary recoveries of chrysin and metabolites were measured. As a result, peak plasma chrysin concentrations were only 3-16 ng ml<sup>-1</sup> with AUCs of 5-193 ng ml<sup>-1</sup> h, whereas chrysin sulphate concentrations were 30-fold higher (AUC 450-4220 ng ml<sup>-1</sup> h). In urine, chrysin and chrysin glucuronide accounted for 0.2-3.1 mg and 2-26 mg, respectively. Most of the dose appeared in faeces as unchanged chrysin. These findings, together with our data, might suggest that unchanged chrysin exists, not in plasma but in intestine, and directly affects the proliferation activity of cryptal cells.

Chrysin is a natural flavonoid that is contained in many plants, honey and propolis. Flavonoids are dietary polyphenols derived from fruits and vegetables (26). Epidemiological observations strongly suggest flavonoids to be preventive in coronary heart disease (27,28), stroke (29) and certain cancers (30). In this study, dietary administration of chrysin reduced the number of mitotic cells and increased the number of apoptotic cells. Recent studies have shown that chrysin induces apoptosis through caspase activation and Akt inactivation in U937 leukemia cells (14), and G2/M cell-cycle arrest in human colon carcinoma SW480 cells (15). Our results are in accordance with those in these *in vitro* studies. Certain components, such as caffeic acid esters and artemillin C, of propolis, which is used as a traditional medicine with a long history in Eastern Europe and Brazil, have been reported to exert antimutagenic and anticarcinogenic effects (31-33). The findings in this study suggest that other components, like chrysin in propolis (0.8 mmol chrysin/100 g of Brazilian propolis) (34), may serve as cancer chemopreventive agents.

In conclusion, this study demonstrates for the first time that dietary administration of chrysin significantly inhibits the development of AOM-induced colonic ACF in rats.

Although the exact mechanisms by which chrysin inhibits ACF development remain to be elucidated, it would appear that the modulation of colon tumorigenesis by chrysin in diet is associated with the alteration of cell proliferation activity and apoptosis.

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## Growth Inhibition and Apoptosis Induction by All-*trans*-conjugated Linolenic Acids on Human Colon Cancer Cells

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**Abstract.** Conjugated linolenic acids (CLN) are geometric and positional isomers of linolenic acid. Growth inhibition and apoptosis induction by  $\alpha$ -eleostearic acid (c9,t11,t13-CLN),  $\beta$ -eleostearic acid (t9,t11,t13-CLN),  $\alpha$ -calendic acid (t8,t10,c12-CLN) and  $\beta$ -calendic acid (t8,t10,t12-CLN) were compared.  $\beta$ -Eleostearic acid and  $\beta$ -calendic acid, which have all-*trans*-conjugated double bonds, exerted stronger growth inhibition and more DNA fragmentation, an indicator of apoptosis induction, in the human colon cancer cells Caco-2 than  $\alpha$ -eleostearic acid and  $\alpha$ -calendic acid with the *cis* configuration. Down-regulation of *bcl-2* and up-regulation of *bax* mRNA by  $\beta$ -eleostearic acid were also greater than by  $\alpha$ -eleostearic acid. Interestingly, the cytotoxic effects of  $\beta$ -eleostearic acid and  $\beta$ -calendic acid were not counteracted completely by  $\alpha$ -tocopherol, whereas the cytotoxic effects of  $\alpha$ -eleostearic and  $\alpha$ -calendic acids were lost in the presence of  $\alpha$ -tocopherol. These results suggest that  $\beta$ -eleostearic and  $\beta$ -calendic acids have signaling pathways different from those of  $\alpha$ -eleostearic and  $\alpha$ -calendic acids and exhibit high potency for reducing the cell viability of Caco-2.

The term "conjugated fatty acids" is generic for polyunsaturated fatty acids with conjugated double bonds in the molecule. Conjugated linoleic acids (CLAs) are known to have many health benefits such as anticancer (1-3), anti-obesity (4, 5) and anti-atherosclerosis effects (6, 7). Conjugated linolenic acids (CLNs), which are geometric and positional isomers of linolenic acid, are also found in high concentrations in some kinds of plant seeds. For example,  $\alpha$ -eleostearic acid (c9,t11,t13-CLN) and  $\alpha$ -calendic acid

(t8,t10,c12-CLN) are contained in bitter melon seed oil (BGO) and pot marigold seed oil, respectively (8). We recently demonstrated that dietary BGO, which contains  $\alpha$ -eleostearic acid, remarkably inhibited the development of azoxymethane-induced colonic aberrant crypt foci (ACF) (9) and adenocarcinoma in F344 rats (10). Furthermore, we and others have reported that free fatty acid prepared from BGO induced apoptosis in colon cancer cells (11, 12).

On the other hand,  $\beta$ -eleostearic acid (t9,t11,t13-CLN) and  $\beta$ -calendic acid (t8,t10,t12-CLN), with all-*trans*-conjugated double bonds, are also known to be contained in some seed oils as minor fatty acids (8). All-*trans*-CLN isomers are also found in mixtures of CLNs chemosynthesized by the alkaline isomerization of linolenic acid (13). Recently, Igarashi and Miyazawa reported that  $\beta$ -eleostearic acid had a stronger antiproliferative effect than  $\alpha$ -eleostearic acid (13). However, there has been no report on apoptosis induction by all-*trans*-CLN isomers in detail, the first study on apoptosis induction by  $\beta$ -eleostearic and  $\beta$ -calendic acids in comparison to  $\alpha$ -eleostearic and  $\alpha$ -calendic acids was performed here. Furthermore, it demonstrated that the effects of the antioxidant  $\alpha$ -tocopherol on reducing the cell viability of Caco-2 differ among all-*trans*-CLN isomers and other isomers with the *cis* configuration.

### Materials and Methods

**Materials.**  $\alpha$ -Eleostearic acid (c9,t11,t13-CLN, >98% purity),  $\beta$ -eleostearic acid (t9,t11,t13-CLN, >97% purity),  $\alpha$ -calendic acid (t8,t10,c12-CLN, >98% purity) and  $\beta$ -calendic acid (t8,t10,t12-CLN, >97% purity) (Figure 1) were purchased from Larodan Fine Chemicals AB, Sweden. WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt) was purchased from Wako Chemical Co. (Tokyo, Japan).

**Cell culture.** The human colon cancer cells line, Caco-2 (ATCC HTB-37) was obtained from the American Type Culture Collection (Rockville, MD, USA). Caco-2 cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acid, 100 U/mL penicillin and

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**Key Words:** Conjugated linolenic acid isomers,  $\beta$ -eleostearic acid,  $\beta$ -calendic acid, apoptosis, Caco-2 cells.

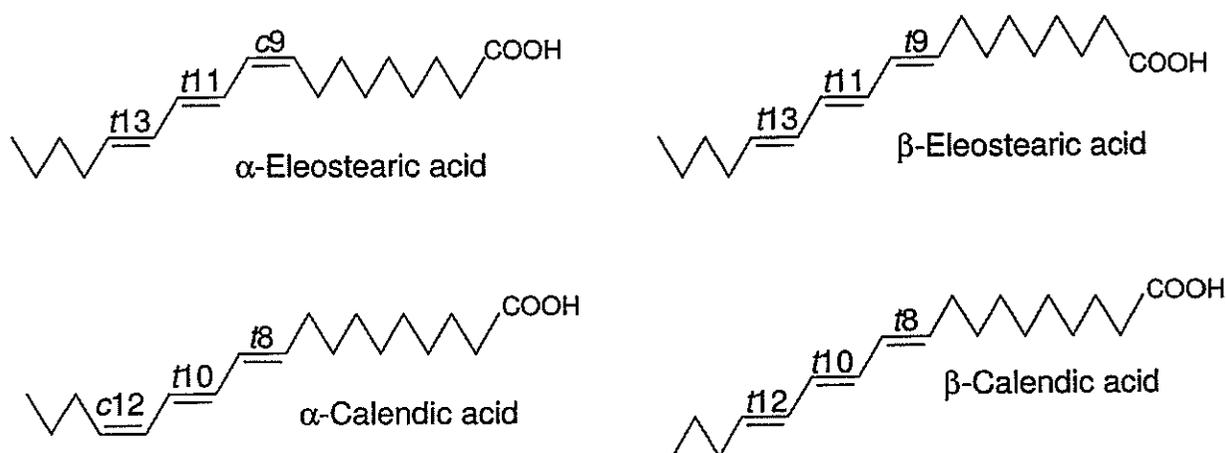


Figure 1. Structure of the conjugated linolenic acid isomers used in this study.

100  $\mu\text{g/mL}$  streptomycin. The cell cultures were maintained in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$  at 37°C.

**Cell viability assay.** Caco-2 cells ( $2 \times 10^3$  cells) were pre-incubated in 96-well plates with 100  $\mu\text{L}$  medium per well for 24 h. Each CLN isomer was dissolved in ethanol and was then added into the culture medium to a final concentration up to 50  $\mu\text{M}$ . The final concentration of ethanol was below 0.1% (v/v). The Caco-2 cells were incubated for 21 h to 45 h in culture medium containing CLN with/without  $\alpha$ -tocopherol at 2.5  $\mu\text{M}$ –50  $\mu\text{M}$ . Then, 10  $\mu\text{L}$  of WST-1 solution (14) were added to each well and the culture plate was incubated for an additional 3 h. The number of viable cells were measured at 450 nm. Viability was expressed as a percentage of the viable cells of the control culture.

**Measurement of DNA fragmentation.** DNA fragmentation was measured as an indicator of apoptotic cells using a commercial kit (Cell Death Detection ELISA<sup>PLUS</sup>, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The assay is based on a quantitative sandwich enzyme immunoassay to detect the histone-associated DNA fragments produced during apoptosis. The cell culture conditions were the same as in the cell viability assay.

**Extraction of total RNA.** Caco-2 cells ( $5 \times 10^5$  cells) were cultivated in 100-mm tissue culture dish for 24 h and each CLN isomer in ethanol was then added to the culture dish. Total RNA was extracted from the Caco-2 cells by an acidic phenol method and further purified by using an RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA), according to the manufacturer's instructions.

**Real-time quantitative RT-PCR analysis.** Total RNA was reverse-transcribed by a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Then, 1  $\mu\text{L}$  of cDNA solution (adequate concentration) was mixed with 1.25  $\mu\text{L}$  TaqMan probe, 12.25  $\mu\text{L}$  TaqMan Master Mix, 10.25  $\mu\text{L}$  water and the quantitative RT-PCR reaction was performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The thermal cycling conditions were as

follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing and extension at 60°C for 1 min. TaqMan probes, *bcl-2* (Hs99999903\_m1), *bax* (Hs00608023\_m1) and  $\beta$ -actin (Hs00180269\_m1), used in this study were purchased from Applied Biosystems.

**Statistical analysis.** The data are expressed as means  $\pm$  SD. The statistical analysis between two groups (Figure 2) was determined using the unpaired Student's *t*-test. Differences with  $p < 0.05$  were considered significant. Statistical analyses between multiple groups were determined by Holm's test ( $p < 0.05$ ) (Figures 3, 4, 5).

## Results

**Effect of CLN isomers on Caco-2 cell viability.** To compare the cytotoxic effects of the CLN isomers, Caco-2 cells were incubated in culture medium containing  $\alpha$ -eleostearic acid,  $\beta$ -eleostearic acid,  $\alpha$ -calendic acid and  $\beta$ -calendic acid (Figure 1). As shown in Figure 2, each CLN isomer exerted a strong cytotoxic effect against the Caco-2 cells in a dose- and time-dependent manner. The  $\beta$ -eleostearic acid and  $\beta$ -calendic acid isomers, in particular, which have the all-*trans* configuration, showed stronger cytotoxic effects than the  $\alpha$ -eleostearic acid and  $\alpha$ -calendic acid isomers, which have the *cis* configuration, at concentrations of 3.125  $\mu\text{M}$  to 12.5  $\mu\text{M}$  after 24-h incubation. At 48-h incubation,  $\beta$ -eleostearic acid and  $\beta$ -calendic acid also remarkably reduced the cell viability compared to  $\alpha$ -eleostearic acid and  $\alpha$ -calendic acid at 3.125  $\mu\text{M}$  and 6.25  $\mu\text{M}$ .

**Comparison of apoptosis induction in Caco-2 cells by CLN isomers.** The DNA fragmentation in Caco-2 cells incubated in culture medium containing CLN isomers was measured as an indicator of apoptosis induction. All of the CLN isomers used in this study induced DNA fragmentation in Caco-2 cells in a dose-dependent manner during 48-h

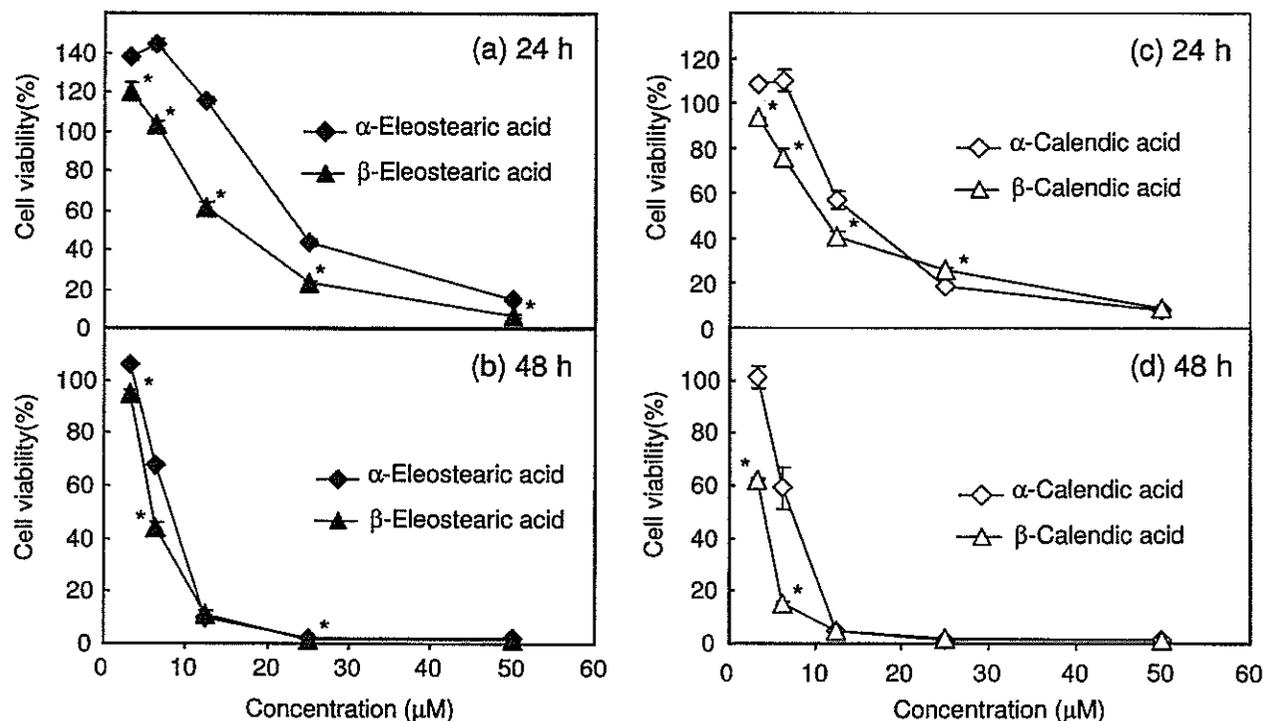


Figure 2. Effect of conjugated linolenic acid isomers on cell viability of Caco-2. Caco-2 cells were incubated in the medium with  $\alpha$ -eleostearic acid,  $\beta$ -eleostearic acid,  $\alpha$ -calendic acid, and  $\beta$ -calendic acid for 24 h or 48 h after 24 h of pre-incubation. The cell viability was measured by WST assay as described in Materials and Methods. The presented data are shown as cell numbers relative to control. All the data are expressed as means  $\pm$  SD of six experiments. \* $p$  < 0.05 vs. Caco-2 cells treated with  $\alpha$ -eleostearic acid and  $\alpha$ -calendic acid at each concentration.

incubation (Figure 3). Relative DNA fragmentation, induced by 6.25  $\mu$ M and 12.5  $\mu$ M  $\beta$ -eleostearic acid, increased to 3.5-fold and 6.9-fold the level of the control after 48 h of incubation, while DNA fragmentation by  $\alpha$ -eleostearic acid was 1.5-fold and 4.9-fold the level of the control, respectively.  $\beta$ -Calendic acid also induced more DNA fragmentation in Caco-2 cells than did  $\alpha$ -calendic acid at 6.25  $\mu$ M, although the DNA fragmentation induced by 12.5  $\mu$ M  $\alpha$ -calendic acid and  $\beta$ -calendic acid was of the same level. Thus,  $\beta$ -eleostearic acid and  $\beta$ -calendic acid induced more DNA fragmentation than did  $\alpha$ -eleostearic acid and  $\alpha$ -calendic acid, respectively. This greater fragmentation corresponds to the reduction of cell viability by each CLN isomer. Furthermore, the down-regulation of *bcl-2* mRNA, which is an anti-apoptotic gene, was observed in the Caco-2 cells treated with 10  $\mu$ M  $\beta$ -eleostearic acid for 24 h (Figure 4). In addition,  $\beta$ -eleostearic acid up-regulated pro-apoptotic *bax* mRNA up to 1.4-fold in Caco-2 cells, while  $\alpha$ -eleostearic acid did not affect *bax* mRNA expression. The greater DNA fragmentation and regulation of apoptosis-related genes corresponded to the reduction of cell viability by each CLN isomer.

*Influence of  $\alpha$ -tocopherol on reducing Caco-2 cell viability by CLN isomers.* In previous studies, we and others have reported that the cytotoxic effect of and apoptosis induction by  $\alpha$ -eleostearic acid are induced through intercellular lipid peroxidation (12, 15, 16). To investigate the mechanisms underlying the cytotoxic effects by all-*trans*-CLN isomers, Caco-2 cells were incubated with CLN isomers and  $\alpha$ -tocopherol. When 25  $\mu$ M  $\alpha$ -tocopherol was added to the culture medium, there was no reduction of cell viability by 50  $\mu$ M  $\alpha$ -eleostearic acid, as found in previous reports (Figure 4) (12, 16). On the other hand, the cytotoxic effect of 50  $\mu$ M  $\beta$ -eleostearic acid was observed even in the presence of 25  $\mu$ M  $\alpha$ -tocopherol, although the viability of the Caco-2 cells was partially restored, to 50% from 0.7%, by the addition of  $\alpha$ -tocopherol (Figure 5).

Furthermore, the effect of  $\alpha$ -tocopherol on the growth inhibition effects of CLN isomers was examined in detail (Figure 6 a). The viability of the Caco-2 cells treated with 25  $\mu$ M  $\beta$ -eleostearic acid increased as the  $\alpha$ -tocopherol concentration increased. However, cell viability reached a plateau at 70% and the cytotoxic effect of  $\beta$ -eleostearic acid was not restored completely by the addition of  $\alpha$ -tocopherol.

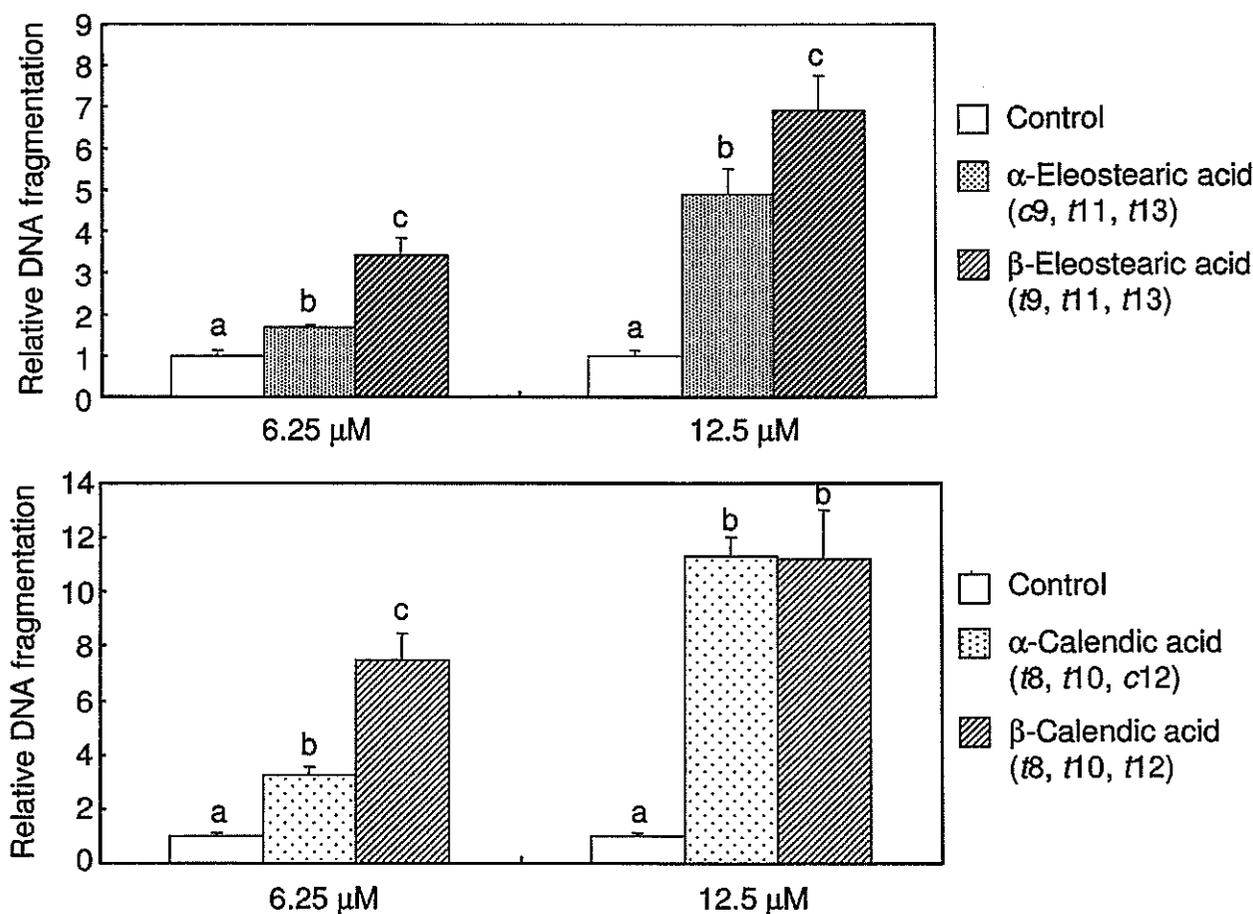


Figure 3. DNA fragmentation in Caco-2 cells treated with CLN isomers. Caco-2 cells were incubated in culture medium with  $\alpha$ -eleostearic acid,  $\beta$ -eleostearic acid,  $\alpha$ -calendic acid and  $\beta$ -calendic acid at 6.25  $\mu$ M or 12.5  $\mu$ M for 48 h after 24 h of pre-incubation. DNA fragmentation was measured by a sandwich enzyme immunoassay using anti-histone antibody and anti-DNA antibody. DNA fragmentation is relative to the assigned control value of 1.0. Values are means  $\pm$ SD, n=3. The values with different letters were significantly different from each other.  $p < 0.05$ .

The cytotoxic effect of 25  $\mu$ M  $\beta$ -calendic acid also remained in the presence of  $\alpha$ -tocopherol (Figure 6 b). In contrast, the cytotoxic effects induced by 25  $\mu$ M  $\alpha$ -eleostearic and  $\alpha$ -calendic acids were lost completely by the addition of  $\alpha$ -tocopherol (Figure 6 a, b).

### Discussion

The results of the present study demonstrated that two all-*trans*-CLN isomers,  $\beta$ -eleostearic and  $\beta$ -calendic acids, have more potent cytotoxic effects and higher levels of apoptosis induction in Caco-2 cells than do  $\alpha$ -eleostearic and  $\alpha$ -calendic acids, CLN isomers with the *cis* configuration. Furthermore,  $\beta$ -eleostearic acid and  $\beta$ -calendic acid showed cytotoxic effects even in the presence of  $\alpha$ -tocopherol, whereas  $\alpha$ -tocopherol counteracted the cytotoxic effects of  $\alpha$ -eleostearic acid and  $\alpha$ -calendic acid.

CLNs are found at high concentrations in the oils of certain kinds of seeds (8).  $\alpha$ -Eleostearic and  $\alpha$ -calendic acids are major fatty acids of bitter gourd seed oil and pot marigold seed oil, respectively. In addition,  $\beta$ -eleostearic acid and  $\beta$ -calendic acid, which have all-*trans*-conjugated double bonds, are found in some kinds of seed oils (8). Moreover, all-*trans*-CLN isomers are produced by the alkaline isomerization of linolenic acid (13). In previous studies, we and others have reported that  $\alpha$ -eleostearic and  $\alpha$ -calendic acids exhibited strong cytotoxic effects on human monocytic leukemia cells (U-937) (15) and colon cancer cells (12, 16). Recently, Igarashi *et al.* reported that  $\beta$ -eleostearic acid inhibits the growth of human tumor cell lines and that its effect is stronger than that of  $\alpha$ -eleostearic acid (13). However, there are no detailed reports about the antiproliferative effects and apoptosis induction by all-*trans*-CLN isomers. In the current study, we demonstrated, for

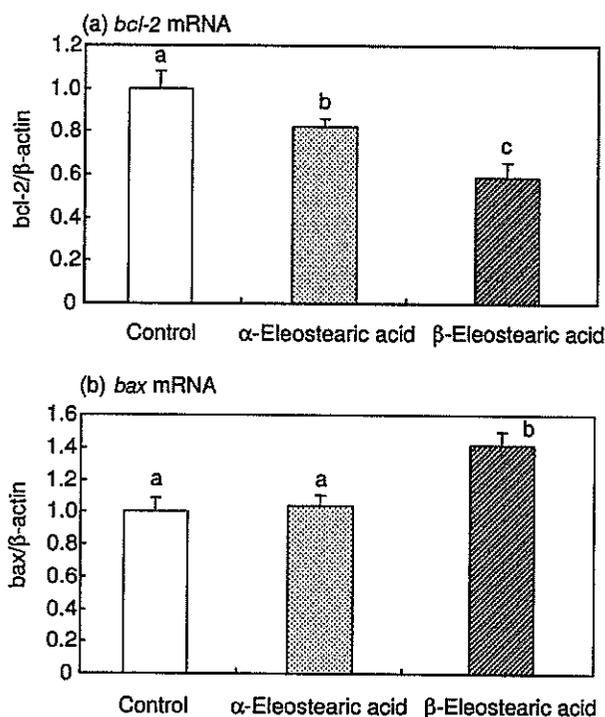


Figure 4. Expression level of *bcl-2* and *bax* mRNA in Caco-2 cells treated with  $\alpha$ -eleostearic acid and  $\beta$ -eleostearic acid. Caco-2 cells were treated with 10  $\mu$ M  $\alpha$ -eleostearic acid or  $\beta$ -eleostearic acid for 24 h. The expression levels of *bcl-2* and *bax* mRNA were estimated by real-time PCR. Data from three independent experiments were normalized to the  $\beta$ -actin mRNA level and are shown as the means  $\pm$  SD. The values with different letters were significantly different from each other.  $p < 0.05$ .

the first time, that two all-*trans*-CLN isomers,  $\beta$ -eleostearic acid and  $\beta$ -calendic acid, induced apoptosis in Caco-2 cells, and that their apoptosis activities were higher than those of  $\alpha$ -eleostearic acid and  $\alpha$ -calendic acid, which have the *cis* configuration. Furthermore, the down-regulation of *bcl-2* mRNA and the up-regulation of *bax* mRNA in Caco-2 cells by  $\beta$ -eleostearic acid were greater than those by  $\alpha$ -eleostearic acid. Bcl-2 and Bax proteins are known to function as anti-apoptotic and pro-apoptotic factors, respectively, in mitochondria (17). These results indicate that the configuration of conjugated double bonds is important with respect to the cytotoxic effect and apoptosis induction in Caco-2 cells. In particular, the all-*trans*-CLN isomers were more effective chemotherapeutic compounds.

The mechanism underlying the cytotoxic effect and apoptosis induction by  $\alpha$ -eleostearic acid involves lipid peroxidation, since the antioxidant  $\alpha$ -tocopherol diminished the growth inhibition and apoptosis induction by  $\alpha$ -eleostearic acid on the colon cancer cell lines. In the present study, we also observed that the cytotoxic effects

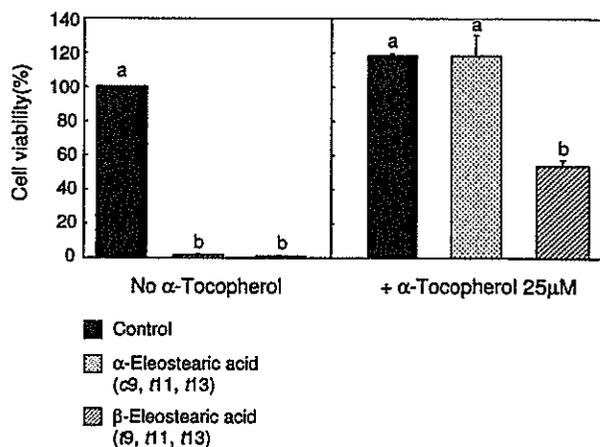


Figure 5. Effect of  $\alpha$ -tocopherol on antiproliferation by  $\alpha$ -eleostearic acid and  $\beta$ -eleostearic acid. Caco-2 cells were treated with 50  $\mu$ M CLN isomers with/without 25  $\mu$ M  $\alpha$ -tocopherol for 48 h. The cell viability was measured by the WST assay described in Materials and Methods. The data represent cell viability expressed as a percentage of the control, which was taken to be 100%. Values are means  $\pm$  SD,  $n = 6$ . The values with different letters were significantly different from each other in the no  $\alpha$ -tocopherol group and the 25  $\mu$ M  $\alpha$ -tocopherol group.  $p < 0.05$ .

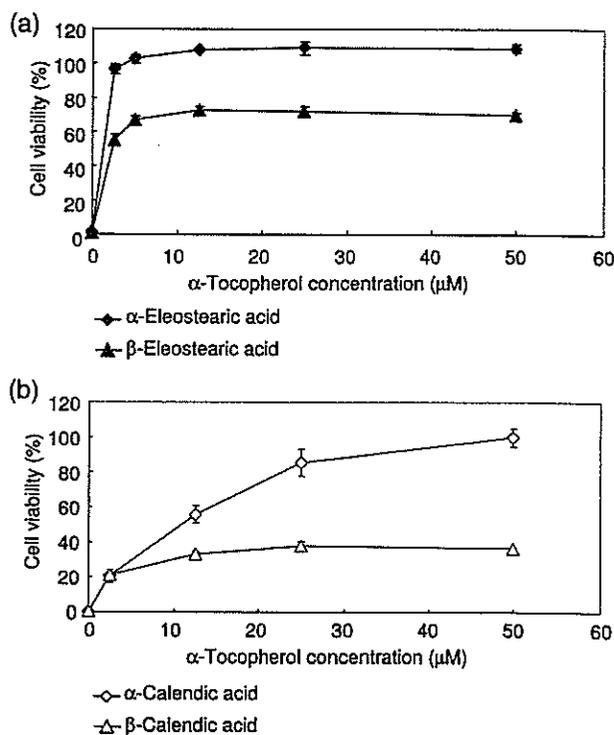


Figure 6. Comparison of antiproliferation by CLN isomers in the presence of  $\alpha$ -tocopherol. (a) Caco-2 cells were treated with 25  $\mu$ M  $\alpha$ -eleostearic acid,  $\beta$ -eleostearic acid and  $\alpha$ -tocopherol after 24 h of pre-incubation. Values are means  $\pm$  SD,  $n = 6$ . (b) Caco-2 cells were treated with 25  $\mu$ M  $\alpha$ -calendic acid,  $\beta$ -calendic acid and  $\alpha$ -tocopherol after 24 h of pre-incubation. The presented data are shown as cell numbers relative to the control. Values are means  $\pm$  SD,  $n = 6$ .