Table 2. Suppressive effects of nobiletin against genotoxicity of NNK in the lung of male *gpt* delta mice

Group number*	Animal I.D.	Total colonies	No. of mutants	*gpt* MF ($\times 10^{-6}$)	Average ±S.D.†	*P*-value‡
1	M001	960,000	21	21.9		
NNK alone	M002	987,000	32	32.4		
	M003	1,320,000	57	43.2		
	M004	876,000	20	22.8		
	M005	1,892,000	23	12.2		
		6,035,000	153	25.4	26.5±11.8	
2	M007	1,156,000	16	13.8		
NNK	M008	991,000	19	19.2		
+Nobiletin	M009	828,000	20	24.2		
(100 ppm)	M010	828,000	23	27.8		
	M011	840,000	12	14.3		
		4,643,000	90	19.4	19.9±6.1	0.147
3	M013	700,000	16	22.9		
NNK	M014	1,404,000	11	7.8		
+Nobiletin	M015	1,052,000	14	13.3		
(500 ppm)	M016	760,000	10	13.2		
	M017	1,000,000	15	15.0		
		4,916,000	66	13.4	14.4±5.4	0.035‖
4	M019	1,028,000	4	3.9		
Nobiletin	M020§	388,000	4	10.3		
(500 ppm)	M021	1,640,000	6	3.7		
alone	M022	708,000	3	4.2		
	M023	972,000	2	2.1		
		4,348,000	15	3.5	3.5±1.0	0.003
5	M024§	705,000	14	19.9		
No treatments	M025	1,410,000	8	5.7		
	M026	1,410,000	5	3.6		
	M027	1,928,000	3	1.6		
	M028	2,032,000	3	1.5		
		6,780,000	19	2.8	3.1±2.0	0.003

*Group 1, mice treated with NNK (2 mg/mouse/day×4 days) alone; Group 2, mice treated with NNK plus nobiletin at a dose of 100 ppm in diet; Group 3, mice treated with NNK plus nobiletin at a dose of 500 ppm in diet; Group 4, mice fed nobiletin at a dose of 500 ppm in diet without NNK treatments; Group 5, mice without treatments with NNK or nobiletin. The Group No. corresponds to Group No. in Fig. 1.

†Average±standard deviation of *gpt* MF of four or five mice.

‡Differences between *gpt* MF of each group and that of Group 1 were tested for statistical significance using a Student's *t*-test.

§Two unusually high *gpt* MF of M020 and M024 were excluded for the calculation of average by the Smirnov-Grubb's outlier test.

‖Statistically significant ($P<0.05$) against Group 1. The values in Groups 4 and 5 are also statistically significant. But the mice in Groups 4 and 5 are not treated with NNK so that the values are not marked with ‖.

against genotoxicity of NNK in the lung of *gpt* delta mice. NNK exposure significantly enhanced the *gpt* MFs in the lung of mice (Tables 1, 2). There was a marked sex difference in the genotoxicity of NNK where females exhibited about twice higher sensitivity than males. This may be due to gender-related differences in the metabolic activation enzymes for NNK (31). The high sensitivity in female than in male mice may be relevant in humans because women are more sensitive to the genotoxic effects of NNK than men (32). Interestingly, dietary administration of nobiletin substantially reduced the *gpt* MFs in both sexes, and the reduction at a dose of 100 ppm in females and 500 ppm in males was statistically significant ($P<0.05$). Administration of nobiletin at 500 ppm also reduced the genotoxicity in females at a similar extent to that observed with nobiletin at 100 ppm. Ikeda *et al.* reported that NNK induces G:C-to-A:T, G:C-to-T:A, A:T-to-T:A, A:T-to-G:C in the lung of *gpt* delta mice (unpublished obervations). Since G:C-to-A:T can activate Ki-*ras* oncogene, the reduction of *gpt* MF may correlate with the reduction of lung tumors (5). Thus, we suggest that nobiletin may be a

chemopreventive agent against NNK-induced lung tumorigenesis in mice. Nobiletin inhibits metastasis (20,21) and suppresses inflammation and promotion (18,33–36). Hence, it may prevent events that occur in multi-step of lung carcinogenesis, i.e., initiation, promotion and progression/metastasis, induced by cigarette smoke. However, certain compounds that can reduce NNK-induced tumors do not necessarily reduce lung tumors in smoke-exposed animals (37). Thus, further examination is needed to evaluate the chemopreventive efficacy of nobiletin against lung tumors induced by cigarette smoke.

In addition to *in vivo* results, we observed reduction of NNK-induced mutations by nobiletin in the present of S9 activation enzymes *in vitro*. Interestingly, nobiletin exhibited a specificity inhibiting the genotoxicity of chemicals in *S. typhimurium*. Although nobiletin inhibited the genotoxicity of NNK, it inhibited the genotoxicity of BP with S9 activation only slightly and did not inhibit the genotoxicity of MNNG without S9 activation. Since MNNG induces O^6-methylguanine leading to G:C-to-A:T mutations (38), we suggest that nobiletin may not enhance the repair activity against O^6-methylguanine or promote error-free translesion bypass across the lesion. Instead, we suggest that nobiletin may suppress the genotoxicity of NNK by inhibiting the activity of CYP (P-450) enzymes involved in the metabolic activation of NNK (39–41). In fact, 8-methoxypsoralen, a specific-inhibitor of CYP2A, similarly suppressed the genotoxicity of NNK in the presence of S9 enzymes (23). The inhibitory effect of nobiletin may be specific to certain CYP enzymes including CYP2A because the genotoxicity of BP, which is activated *via* CYP1A1 (42), was weakly inhibited by nobiletin. However, since both nobiletin and 8-methoxypsoralen inhibited the genotoxicity of NNK only by 50%, we suggest that other CYP enzymes may be responsible for the remaining genotoxicity of NNK in the S9 enzymes. Although nobiletin did not effectively affect the genotoxicity of BP in the present study, Conney *et al.* (43) observed that nobiletin stimulates human liver microsomes and activates both the hydroxylation of BP and the metabolism of aflatoxin B_1 to mutagens. Nobiletin also stimulates oxidative metabolism of zoxazolamine by rat liver microsomes (44) and acetaminophen by human liver microsome (45). These reports suggest that nobiletin has a potential to modulate CYP enzyme activities.

In summary, we examined the chemopreventive efficacy of nobiletin against the genotoxicity of NNK in the lung of female and male *gpt* delta mice. Dietary administration of nobiletin significantly reduced the genotoxicity of NNK in both sexes. In addition, the chemical was able to reduce NNK-induced genotoxicity in *S. typhimurium* YG7108 in the presence of S9 activating enzymes. Our findings suggest that nobiletin could inhibit the activities of certain CYP enzymes involved in the metabolic activation of NNK, thereby suppressing the genotoxicity in the lung of mice.

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Catalpa seed oil rich in 9*t*,11*t*,13*c*-conjugated linolenic acid suppresses the development of colonic aberrant crypt foci induced by azoxymethane in rats

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Abstract. Catalpa (*Catalpa ovata*) seed oil (CPO) is a unique oil that contains a high amount of 9*trans*,11*trans*,13*cis*-conjugated linolenic acid. In the present study, we investigated whether dietary administration with CPO affects the development of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) in male F344 rats to elucidate its possible cancer chemopreventive efficiency. Also, the effect of CPO on the fatty acid composition of liver tissue and colonic mucosa, the serum levels of total cholesterol and triglyceride, and the mRNA expression of cyclooxygenase (COX)-2 in the colonic mucosa were measured. In addition, the cell proliferation activity and apoptotic index in the colonic mucosa were estimated immunohistochemically. Animals were given two weekly subcutaneous injections of AOM (20 mg/kg body weight). They also received the experimental diet containing 0.01%, 0.1% or 1% CPO for 4 weeks, starting one week before the first dosing of AOM. AOM exposure produced a substantial number of ACF (99±28) at the end of the study (week 4). Dietary administration of CPO reduced the number of ACF (AOM + 0.01% CPO, 32±11, P<0.001; AOM + 0.1% CPO, 35±18, P<0.001; AOM + 1% CPO, 18±10, P<0.001). 9*t*,11*t*-conjugated linoleic acid was detected in the liver tissue and colonic mucosa of rats fed the CPO-containing diet. Additionally, dietary administration with CPO decreased the serum triglyceride level and the expression of COX-2 mRNA in the colonic mucosa. The indices of cell proliferation and apoptosis in the colonic mucosa of rats treated with AOM and 1% CPO have significant differences when compared with the AOM alone group. These findings suggest the possible chemopreventive activity of CPO in the early phase of colon carcinogenesis.

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Key words: catalpa seed oil, conjugated linolenic acid, aberrant crypt foci, inhibition, rat, colon

Introduction

Colon cancer is one of the leading causes of cancer deaths in Asia and Western countries (1,2). Therefore, it is a major public health problem around the world. Dietary factors, including a high fat content, influence colon cancer development (1,2). Intake of n-3 polyunsaturated fatty acid (PUFA) is reported to prevent colorectal carcinogenesis (3,4), while excessive consumption of n-6 PUFA or saturated fatty acids could promote colon cancer development (5). The type of dietary fat consumption is thus important for development of colonic malignancy.

Recently, conjugated fatty acids (CFAs) have received a great deal of attention because of their numerous beneficial biological effects including cancer preventive property (6-8). CFAs refer to a group of positional and geometric isomers of PUFAs containing conjugated double bonds. One of the CFAs, conjugated linoleic acid (CLA), is well-known for its biological effects. Regarding the inhibitory effect of cancer, CLA can inhibit chemically-induced skin, mammary, forestomach, and colon tumorigenesis in rodents (9-12). Although CLA is present in certain foods such as milk fat and meats derived from ruminant animals, the content is less than 1% (13).

On the other hand, some plant seed oils contain a large amount of conjugated linolenic acid (CLN). α-Eleostearic acid (9*c*,11*t*,13*t*-CLN) in tung and bitter melon seed oil (BMO) are present at 67.7% and 56.2%, respectively (14). The seed oils of pomegranate, catalpa, and pot marigold contain 83.0% punicic acid (9*c*,11*t*,13*c*-CLN), 42.3% catalpic acid (9*t*,11*t*,13*c*-CLN), and 62.2% calendic acid (8*t*,10*t*,12*c*-CLN), respectively (14). As for the biological activity of CLN, dietary CLN produced by alkaline isomerization of linolenic acid (LN) reduces fat content in the body (15). Purified punicic acid has a hypolipidemic effect (16). Additionally the anti-obese property of pomegranate seed oil (PGO) has been reported. (17). Also we and other researchers have shown the cytotoxic effect of PGO and tung oil on a variety of human cancer cell lines, including colon cancer cells (18,19).

Table I. Fatty acid composition of CPO.

Fatty acid (wt%)	
16:0	2.8
18:0	2.2
18:1 n-9	7.6
18:2 n-6	42.5
18:3 n-3	0.6
9*c*,11*t*,13*c*-CLN	0.1
9*c*,11*t*,13*t*-CLN	0.1
9*t*,11*t*,13*c*-CLN	40.2
Others	3.9

Regarding the *in vivo* studies, CLN derived from perilla oil suppresses chemically-induced mammary adenocarcinomas in rats (10). We also found that dietary BMO inhibits the development of aberrant crypt foci (ACF) (20) that are precursor lesions of colon cancer (21). In addition, our recent studies demonstrated that BMO and PGO suppressed azoxymethane (AOM)-induced colon carcinogenesis in rats (12,22). Regarding catalpa seed oil (CPO), we reported the cytotoxic effect of CPO on SV40-transformed Balb 3T3 A31 and human monocytic leukemia cell lines (18), but there are no *in vivo* studies on the effect of CPO on carcinogenesis.

In the present study, we investigated the influence of CPO in the development of AOM-induced ACF to elucidate the modifying effect of CPO on rat colon carcinogenesis. Additionally, we analyzed the lipid composition of liver tissue and colonic mucosa and measured the serum concentrations of total cholesterol and triglyceride to understand the possible mechanisms by which CPO could modify the occurrence of the lesions. Since overexpression of cyclooxygenase-2 (COX-2) is involved in colon carcinogenesis and certain cyclooxygenase inhibitors are likely to be useful as colon cancer chemopreventive agents (23-26), the effects of CPO on the expression of COX-2 in the non-lesional colonic mucosa were investigated. Also, biomarkers such as proliferating cell nuclear antigen (PCNA)-labeling index and apoptotic index were measured immunohistochemically in colonic mucosa, since BMO exerted an inhibitory effect on ACF via reduction of the PCNA index and induction of apoptosis in our previous study (20).

Materials and methods

Animals, chemicals, and diets. Male F344 rats (Charles River Japan, Inc., Tokyo, Japan) aged 4 weeks were used. The animals were maintained at Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. They were housed in plastic cages (4 rats/cage) with free access to tap water and diet, under controlled conditions of humidity (50±10%), lighting (12-h light/dark cycle), and temperature (23±2°C). They were quarantined for 7 days and randomized by body weight into experimental and control groups. AOM for ACF induction was purchased from Sigma Chemical Co. (St. Louis, MO, USA). AIN-76A diet

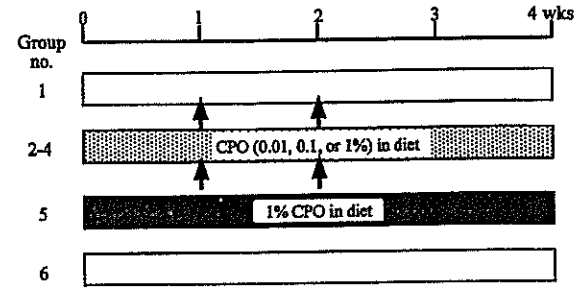

Figure 1. Experimental protocol. White bar, basal diet; dotted bar, CPO (0.01, 0.1, and 1%); dashed bar, CPO 1%; arrow, AOM, 20 mg/kg bw s.c.

(LSG Corporation, Tokyo, Japan) was used as basal diet throughout the study. Seeds of catalpa (*Catalpa ovata*) were kindly donated from Tohoku Seed Co. (Tochigi, Japan). Seed oil was extracted by *n*-hexane after crushing with an electric mill. Determination of fatty acid profile of the total lipids in CPO (Table I) was carried out according to the methods described previously (20).

Experimental procedure. A total of 40 male F344 rats were divided into five experimental groups and a control group (Fig. 1). Animals in groups 1 through 4 were initiated with AOM by two weekly subcutaneous injections (20 mg/kg body weight). Rats in groups 1 and 6 were fed the basal diet containing 5% corn oil. The diets for groups 2 and 3 were replaced by 0.01% and 0.1% CPO in the 5% corn oil, respectively. Groups 4 and 5 were given the diet containing 1% CPO and 4% corn oil. These diets were given to rats for 4 weeks, starting one week before the first dosing of AOM. All rats were freely available for diet and tap water. All experimental diets containing CPO were prepared weekly in our laboratory and stored at -20°C under a nitrogen atmosphere in airtight containers for no longer than a week. Rats were provided with the diet every day and the peroxide value of the lipids in the fresh diets was less than 3.0 meq/kg lipid. The rats were sacrificed under ether anesthesia at week 4 and underwent careful necropsy, with emphasis on the colon, liver, kidney, lung, and heart. The colons of five rats each from groups 1 through 4 and those of two rats each from groups 5 and 6 were fixed in 10% buffered formalin for assessing the occurrence of colonic ACF. The colons of the remaining rats were used for determining the expression of COX-2 protein and lipid analysis in colonic mucosa. The liver was weighed and the caudate lobe was removed and fixed in 10% buffered formalin for histological examination. Remaining lobes of the livers of all rats were used for analyses of fatty acid composition. All other tissues were fixed in 10% buffered formalin and submitted to histological examination.

Determination of ACF. The presence of ACF was determined according to the standard procedures that are routinely used in our laboratory (27). At necropsy, the colons were flushed with saline, excised, cut open longitudinally along the main axis, and then washed with saline. They were cut, placed on the filter paper, with their mucosal surface up, and then fixed in 10% buffered formalin for at least 24 h. Fixed colons were stained with methylene blue (0.5% in distilled water) for

Table II. Body, liver, and relative liver weights in each group.

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)	209±11[a]	8.4±0.7	4.03±0.33
2	AOM + 0.01% CPO (8)	198±5	7.2±0.7	3.61±0.38
3	AOM + 0.1% CPO (8)	197±13	6.9±0.9[b]	3.51±0.24
4	AOM + 1% CPO (8)	203±10	8.4±1.3	4.12±0.46
5	1% CPO (4)	204±8	7.8±0.5	3.80±0.30
6	No treatment (4)	1 99±11	8.5±1.3	4.25±0.46

[a]Mean ± SD. [b]Significantly different from group 1 by Bonferroni Multiple Comparisons test ($P<0.05$).

20 sec, dipped in distilled water, and placed on a microscope slide for counting ACF.

Lipid extraction and analysis. Tissue lipids were extracted by the Folch method using chloroform/methanol (2:1, v/v) (28). Fatty acid methyl esters were prepared according to the method by Prevot and Mordret (29). Fatty acid methyl esters were analyzed by GC-FID (SHIMADZU GC-14B gas chromatograph, Shimadzu Seisakusho Co., Ltd., Kyoto, Japan) equipped with an Omegawax 320 capillary column (30 m x 0.32 mm I.D.). Peaks were identified by comparison with fatty acid standards (Nu-chek-Prep, MN, USA), and area and its percentage for each resolved peak were analyzed using Shimadzu Chromatopac C-R3A integrator (Shimadzu Seisakusho Co., Ltd.). The identification of CLA and/or CLN isomers was confirmed using GC-mass spectrometry after conversion of the methyl esters to dimethyloxazoline derivatives (30).

Measurements for the level of serum cholesterol and triglyceride. Serum cholesterol and triglyceride levels in rats were measured by enzymatic method using an Ekudia-L-Eiken kit according to the manufacturer's protocol (Eiken Chemical Co., Ltd., Tokyo, Japan).

Determination of COX-2 mRNA level in colonic mucosa by real-time PCR. For quantitative real-time PCR, total RNA was extracted from colonic mucosa using a Qiagen RNeasy mini kit (Qiagen, CA, USA) after homogenization using a QiAshredder column (Qiagen), and stored at -80°C. Total RNA was reverse transcribed by the High Capacity cDNA Archive kit (Applied Biosystems, CA, USA). cDNA was subjected to quantitative real-time PCR using TaqMan gene expression assay (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems). An ABI PRISM 7000 system (Applied Biosystems) was used for the reaction and detection of the expression of COX-2 and ß-actin mRNA. PCR amplification was performed in a total volume of 25 μl containing 11.25 μl cDNA template, 12.5 μl of 2X TaqMan Universal PCR Master Mix, and 1.25 μl of 20X TaqMan gene expression assay. For each reaction the AmpErase UNG and AmpliTaq Gold Enzyme were activated at 50°C for 2 min and 95°C for 10 min, respectively. Amplification was then performed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Immunohistochemistry. Immunohistochemistry for the PCNA and apoptotic nuclei was performed on 4-μm-thick paraffin-embedded sections from colons of rats 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°C, deparaffinized in xylene, and rehydrated through graded ethanol 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, PCNA-immunohistochemistry was performed according to the method described by Watanabe *et al* (31). Apoptotic index was also evaluated by immunohistochemistry for single-stranded DNA (ssDNA) (31). Sections were treated for 40 min at room temperature with 2% BSA and incubated overnight at 4°C with primary antibodies. Primary antibodies included anti-PCNA mouse monoclonal antibody (diluted 1:50; PC10, Dako Japan) and anti-ssDNA rabbit polyclonal antibody (diluted 1:300, Dako Japan). Horseradish peroxidase activity was visualized by treatment with H_2O_2 and 3,3'-diaminobenzidine for 5 min. At the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck Ltd., 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 two primary antibodies used were examined on all sections using a microscope (Olympus BX41, Olympus Optical Co., Ltd., 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.

Statistical evaluation. Where applicable, data were analyzed using one-way ANOVA with Bonferroni correction test with $P<0.05$ as the criterion of significance.

Results

General observation. Body, liver, and relative liver weights (g/100 g body weight) in all groups are shown in Table II. All animals remained healthy throughout the experimental period. Food consumption (g/day/rat) did not differ significantly among the groups (data not shown). At the end of the study,

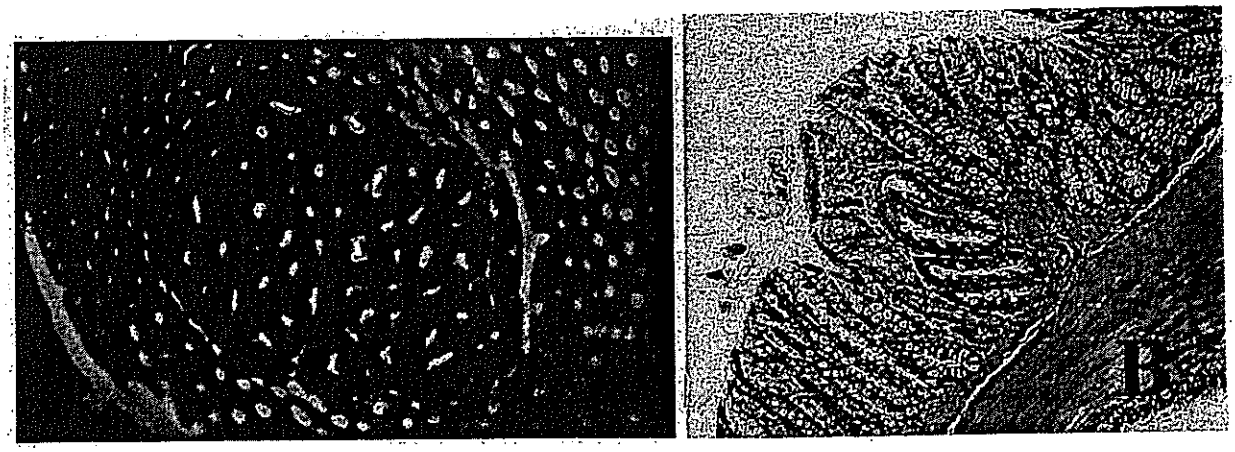

Figure 2. Morphology of representative ACF found in a rat from group 1. (A) ACF on methylene-blue-stained colonic mucosa; and (B) ACF on hematoxylin and eosin-stained section. Original magnification, (A) x4; and (B) x10.

Table III. Effect of CPO on AOM-induced ACF formation in male F344 rats.

Group no.	Treatment (no. of rats examined)	No. of ACF/colon	No. of ACs/colon	No. of ACs/focus	% of ACF containing 4 or more ACs
1	AOM alone (5)	99±28[a]	295±66	3.01±0.20	32.13±4.28
2	AOM + 0.01% CPO (5)	32±11[b]	50±17[b]	1.57±0.10[b]	1.25±2.80[b]
3	AOM + 0.1% CPO (5)	35±18[b]	60±35[b]	1.70±0.21[b]	1.30±1.78[b]
4	AOM + 1% CPO (5)	18±10[b]	32±18[b]	1.80±0.15[b]	1.62±2.25[b]
5	1% CPO (2)	0	0	0	0
6	No treatment (2)	0	0	0	0

[a]Mean ± SD. [b]Significantly different from group 1 by Bonferroni Multiple Comparisons test ($P<0.001$).

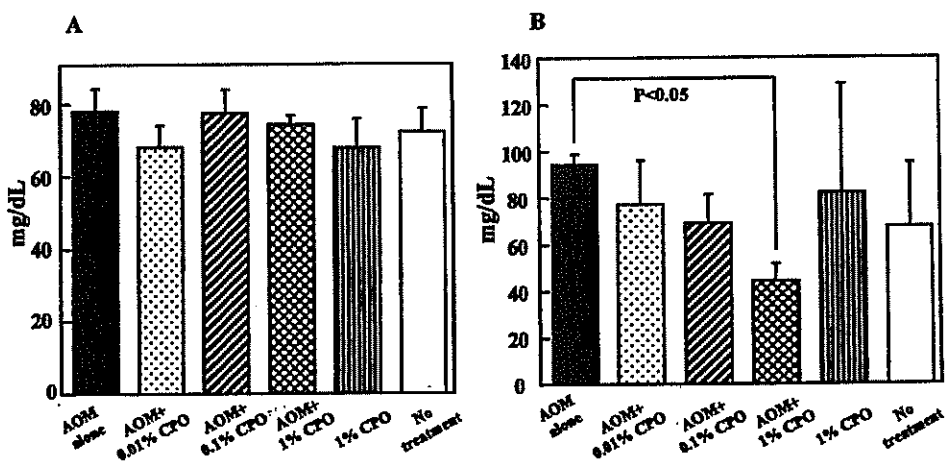

Figure 3. Effect of CPO on total cholesterol and triglyceride in serum. (A) Total cholesterol level, (B) triglyceride level.

there were no significant differences in the mean body weight among the groups. Although the liver weight of group 3 was statistically lower than that of group 1 ($P<0.05$), the mean relative liver weights did not show significant differences among the groups.

ACF analysis. The data on colonic ACF (Fig. 2) formation are summarized in Table III. All rats belonging to groups 1 through 4, which were given AOM, developed ACF. When compared to the mean number of ACF/colon in group 1 (AOM alone, 99±28), the dietary administration of 0.01%

Table IV. Effect of CPO diets on fatty acid composition of liver lipids.

Group	Treatment	Fatty acid (wt%)									
no.	(no. of rats examined)	16:0	16:1 n-7	18:0	18:1 n-9	18:1 n-7	18:2 n-6	20:4 n-6	22:5 n-6	22:6 n-3	9*c*,11*t*-CLA	9*t*,11*t*-CLA
1	AOM alone (8)	23.6±0.8[a]	5.7±1.1	13.6±1.2	13.9±2.5	5.8±0.2	10.8±1.4	17.3±1.4	1.3±0.1	2.5±0.4	N.D.	N.D.
2	AOM +0.01% CPO (8)	24.1±1.7	5.2±1.3	12.6±1.3	15.7±2.3	4.5±0.5	11.4±1.6	17.1±2.3	0.9±0.1	3.1±0.5	0.01±0.0	0.05±0.0
3	AOM +0.1% CPO (8)	23.7±1.6	5.4±1.3	13.2±1.5	15.2±2.3	4.9±0.7	10.4±1.1	17.6±2.7	1.1±0.2	2.9±0.7	0.01±0.0	0.07±0.0
4	AOM + 1% CPO (8)	22.9±1.0	4.5±0.2	14.6±0.8	12.6±1.5	5.3±0.7	11.3±0.1	17.0±1.1	1.1±0.2	3.0±0.1	N.D.	0.52±0.1
5	1% CPO (4)	25.3±1.3	5.3±0.7	13.4±0.9	16.2±1.5	3.3±0.2	11.1±0.6	16.5±2.0	0.5±0.2	3.3±0.3	N.D.	0.04±0.0
6	No treatment (4)	26.9±1.2	6.7±0.7	12.2±1.4	19.4±2.1	3.4±0.3	9.4±0.2	15.0±2.0	0.5±0.1	2.8±0.4	N.D.	N.D.

[a]Mean ± SD. N.D., not detected.

Table V. Effect of CPO diets on fatty acid composition of colonic mucosa.

Group	Treatment	Fatty acid (wt%)								
no.	(no. of rats examined)	14:0	16:0	16:1 n-7	18:0	18:1 n-9	18:1 n-7	18:2 n-6	20:4 n-6	9*c*,11*t*-CLA	9*t*,11*t*-CLA
1	AOM alone (5)	1.5±0.0[a]	28.3±1.2	8.9±1.2	3.1±0.5	28.0±0.6	4.0±0.3	21.0±0.4	1.3±0.8	0.08±0.0	0.09±0.0
2	AOM + 0.01% CPO (5)	1.4±0.0	30.0±0.3	9.1±0.3	3.3±0.5	29.3±0.8	3.7±0.1	18.4±0.8	1.0±0.7	0.06±0.0	0.11±0.0
3	AOM + 0.1% CPO (5)	1.4±0.0	29.2±0.9	9.6±0.9	3.5±0.5	27.7±0.7	3.8±0.3	18.4±1.5	1.6±0.6	0.06±0.0	0.30±0.0
4	AOM + 1% CPO (5)	1.5±0.0	28.9±1.1	9.9±1.1	2.9±0.3	26.2±0.4	3.7±0.0	18.8±1.2	1.2±0.4	0.09±0.0	2.29±0.2
5	1% CPO (2)	1.5±0.1	30.2±0.6	10.5±0.6	2.7±0.2	26.8±0.3	3.7±0.2	18.1±0.7	0.8±0.4	0.09±0.0	2.44±0.3
6	No treatment (2)	1.6±0.0	31.1±0.3	10.1±0.3	3.2±0.1	29.6±0.3	3.4±0.1	17.4±0.9	1.0±0.1	0.07±0.0	0.09±0.0

[a]Mean ± SD.

(group 2, 32±11, P<0.001), 0.1% (group 3, 35±18, P<0.001), and 1% (group 4, 18±10, P<0.001) CPO significantly reduced the number of ACF: 68% inhibition in group 2, 65% inhibition in group 3, and 82% inhibition in group 4. Furthermore, significant decreases were found in the number of aberrant crypts (ACs) per colon (P<0.001) and the number of ACs/focus (P<0.001) in groups 2 through 4 when compared to those in group 1. Also the percentages of ACF consisting of more than 4 ACs in groups 2 (1.25±2.80, P<0.001), 3 (1.30±1.78, P<0.001), and 4 (1.62±2.25, P<0.001) were significantly smaller than that of group 1 (32.13±4.28). In groups 5 and 6, there was no microscopically observable change, including ACF, in the colonic mucosa.

Lipid analysis. The fatty acid profiles of the lipids from the liver tissue and colonic mucosa are shown in Tables IV and V, respectively. CPO diets contained ~40% of catalpic acid (9*t*,11*t*,13*c*-CLN), however it was not detected in these tissues of rats fed CPO-containing diets at three different doses. On the other hand, the contents of CLA (9*t*,11*t*-18:2) in the same tissues were elevated in a dose-dependent manner. Although the CPO diets contained >40% of linoleic acid (LA), the amount of LA in the groups administered the CPO-containing diet was insignificant compared with that in the groups fed the diet without CPO.

Serum concentration of total cholesterol and triglycerides. Serum concentrations of total cholesterol and triglycerides are summarized in Fig. 3. The total cholesterol level in the AOM + 0.01% CPO group (67.4±6.0 mg/dl) was lower than that in the AOM alone group (77.4±6.2 mg/dl) without statistical significance. Serum triglyceride levels of rats that were fed the AOM + CPO diet (the AOM + 0.01% CPO group: 77.2±18.7 mg/dl; the AOM + 0.1% CPO group: 69.0±12.7 mg/dl; and the AOM + 1% CPO group: 44.6±7.6 mg/dl) were reduced dose dependently, when compared to the AOM alone group (94.6±4.0 mg/dl). A significant difference (P<0.05) was detected between the AOM + 1% CPO and AOM alone group.

Expression of COX-2 mRNA levels in colonic mucosa. As illustrated in Fig. 4, expression of COX-2 mRNA level was

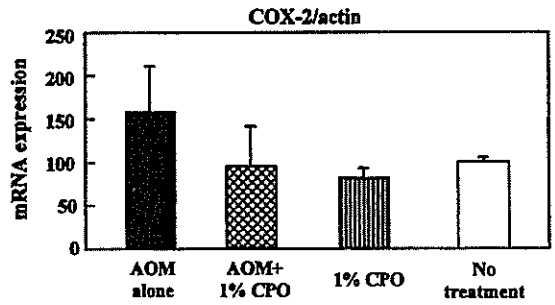

Figure 4. Effect of CPO diet on COX-2 mRNA expression in rat colon mucosa.

Table VI. PCNA and apoptosis indices in rat colonic mucosa.

Group no.	Treatment (no. of rats examined)	PCNA-labeling index (%)	Apoptotic index (%)
1	AOM alone (5)	24.6±5.6[a]	3.8±0.8
2	AOM + 0.01% CPO (5)	18.8±4.1	5.4±2.3
3	AOM + 0.1% CPO (5)	17.4±2.9	6.8±1.5
4	AOM + 1% CPO (5)	15.2±3.2[b]	8.2±1.3[c]

[a]Mean ± SD. [b,c]Significantly different from group 1 by Bonferroni Multiple Comparisons test ([b]P<0.05 and [c]P<0.01).

up-regulated by ~1.5-fold in the colonic mucosa of the AOM alone group when compared with the untreated group (group 6). On the other hand, rats that received AOM and CPO-containing diet showed a low expression level of COX-2 mRNA.

Immunohistochemistry for PCNA and ssDNA in colonic mucosa. As summarized in Table VI, the PCNA-labeling index of colonic mucosa in groups 2 (18.8±4.1), 3 (17.4±2.9), and 4 (15.2±3.2) was smaller than that in group 1 (24.6±5.6). Apoptotic index measured by ssDNA immunohistochemistry in groups 2 (5.4±2.3), 3 (6.8±1.5), and 4 (8.2±1.3) was greater than in group 1 (3.8±0.8). For both PCNA-labeling index ($P<0.05$) and apoptotic index ($P<0.01$), statistically significant differences were found in group 4.

Discussion

The results described here clearly indicate that dietary administration with CPO that contains a large amount of catalpic acid (9*t*,11*t*,13*c*-CLN) significantly reduced AOM-induced rat ACF formation at any dose level (0.01%, 0.1%, or 1%) as compared with the rats injected with AOM alone. Also, animals fed the diets containing CPO showed no adverse effects on food intake or growth rate and no histological alterations in any organs. These findings may suggest that dietary CPO suppresses the early phase of chemically-induced colon carcinogenesis. Previously we reported that dietary administration with 9*c*,11*t*,13*t*-CLN caused a significant reduction in the frequency of ACF (19% reduction by 0.01% 9*c*,11*t*,13*t*-CLN, 36% reduction by 0.1% 9*c*,11*t*,13*t*-CLN, and 63.0% reduction by 1% 9*c*,11*t*,13*t*-CLN) (20). In the present study, the inhibition rates of the total number of ACF at 0.01, 0.1, and 1% CPO were 68, 65, and 82%, respectively. This may suggest that the distinction of geometric isomers of CLN might have an explanation for such differences of inhibition rate. Despite CPO containing a significant amount of LA that influences colorectal cancer (5), colonic ACF was suppressed by CPO. Our findings may suggest that *t*9,*t*11,*c*13-18:3 in CPO is a good natural chemopreventive agent against colon carcinogenesis.

COX enzymes play a central role in the conversion of arachidonic acid (AA) to prostaglandins. One of the COX-2 reaction products, PGE2, is known to lead to the induction of cell proliferation and the inhibition of apoptosis which favor tumor development (23,26). Suppression of the enzyme COX is suggested to be the potential mechanism for inhibition of carcinogenesis. In colon carcinogenesis, overexpression of COX-2 was observed in ACF, adenomas, and adenocarcinomas (32), suggesting that the over-expression of COX-2 contributes to the growth of precursor lesions and tumors and their progression. In fact, treatment with the selective COX-2 inhibitor celecoxib gave us promising results in the prevention of colorectal cancer (26). Also Rao *et al* (24) demonstrated that celecoxib significantly reduced AOM-induced rat colon ACF. In the current study, dietary administration with CPO reduced COX-2 mRNA expression in the colonic mucosa, and this may account for lowering the number of colonic ACF. Since CLA causes down-regulation of COX-2 activity (33), accumulated CLA in the colonic mucosa might also contribute to reducing the expression of COX-2 mRNA.

In the current study, dietary administration with CPO decreased cell proliferation activity and increased apoptosis index in the AOM-induced rat colonic mucosa. Also, down-expression of anti-apoptosis protein, bcl-xL, was found in the colonic mucosa of rats treated with AOM and CPO (data not shown). Previously, we reported that BMO has the ability to decrease the PCNA-labeling index and enhance the apoptosis-index (20). Thus, the inhibitory effect of CPO on ACF might partly be due to modulation of cell proliferation and apoptosis. Since inhibition of COX-2 expression results in resistance to cell proliferation and increased apoptosis (23,26), the reduction of COX-2 mRNA expression in the present study might contribute to the modulation effect of CPO on cell proliferation and apoptosis.

We found a dose-dependent accumulation of *t*9,*t*11-18:2 in the colonic mucosa and liver tissue of rats that were fed CPO. Also the contents of *c*9,*t*11-18:2 in the colonic mucosa and liver tissue of rats given PGO that contained over 70% of *c*9,*t*11,*c*13-18:3 were elevated in a dose-dependent manner (12). Others reported that CLA generated in rats after *c*9,*t*11,*t*13-18:3 administration was confirmed to be *c*9,*t*11-18:2 (34). We speculated that *t*9,*t*11,*c*13-18:3 rich in CPO was saturated at the Δ13 position and converted to *t*9,*t*11-18:2 in the current study. We and others reported that CLA inhibits the occurrence of chemically-induced colonic ACF (35) and tumor (12) in rats. Also *t*9,*t*11-18:2 can suppress the growth of human cancer cells (36). Therefore, *t*9,*t*11-18:2

converted from *t*9,*t*11,*c*13-18:3 might contribute to prevention of the development of colonic preneoplasms in the present study. CPO contains ~40% LA besides catalpic acid. After intake of LA, it can be oxidized, stored in triacylglycerides, incorporated into membranous phospholipids, or elongated and desaturated to more unsaturated fatty acids such as γ-linolenic acid (LN), dihomo-γ- LN, and AA. Excess of n-6 PUFA consumption is one of the causes of colorectal cancer development (5). Administration of an LA-enriched diet also enhanced chemically-induced rat ACF occurrence and multiplicity (37). In the current study, the amounts of LA and AA of colonic mucosa and liver lipids in groups 2 through 4 did not significantly differ from those in group 1. These modifying effects of CPO on fatty acid profile in colonic mucosa or liver lipid might partly influence its inhibitory ability in ACF formation.

Serum levels of triglycerides and cholesterol have been shown to be positively associated with colon carcinogenesis (38,39). Niho *et al* found that serum levels of triglycerides in Min mice are dramatically increased compared to the wild-type and the increase contributes to the growth of small intestinal polyps (40). They also demonstrated that a peroxisome proliferator-activated receptors (PPAR)γ ligand suppresses both serum triglyceride level and intestinal polyp formation in Min mice (40). A synthetic ligand for PPARγ can inhibit AOM-induced rat colonic ACF (41) and colitis-related mouse colon cancer development (42). These findings suggest that activation of PPARγ is beneficial for colon cancer prevention. Since BMO containing *c*9,*t*11,*t*13-18:3 and PGO rich in *c*9,*t*11,*c*13-18:3 can up-regulate PPARγ (12,43), CPO might be a natural ligand of PPARγ. Feeding with CLA also increases the expression of PPARγ protein as compared to the basal diet (12). Since oxidative metabolites of LA, including 13-hydroxyoctadecadienoic acid and 13-oxooctadecadienoic acid are reported to activate PPARγ (44), it may be possible that accumulated CLA or linoleate metabolites activated PPARγ in this study. Although we did not determine PPARγ expression in the colon, possible modulatory effects of CPO may partly contribute to its inhibitory effect on ACF occurrence.

In conclusion, the findings described here demonstrate for the first time that dietary administration of CPO rich in catalpic acid (9*t*,11*t*,13*c*-CLN) significantly inhibited the development of AOM-induced ACF in rats. Although the exact mechanisms by which CPO inhibits colonic early preneoplastic lesions remain to be elucidated, it would be worthwhile to test the cancer chemoprevention ability of CPO using a long-term colon carcinogenesis model.

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Potent inhibitory effect of *trans*9, *trans*11 isomer of conjugated linoleic acid on the growth of human colon cancer cells

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Abstract

This study compared the growth inhibitory effects of pure conjugated linoleic acid (CLA) isomers [*cis*(c)9,c11-CLA, c9,*trans*(t)11-CLA, t9,t11-CLA, and t10,c12-CLA] on human colon cancer cell lines (Caco-2, HT-29 and DLD-1). When Caco-2 cells were incubated up to 72 h with 200 μM, each isomer, even in the presence of 10% fetal bovine serum (FBS), cell proliferation was inhibited by all CLA isomers in a time-dependent manner. The strongest inhibitory effect was shown by t9,t11-CLA, followed by t10,c12-CLA, c9,c11-CLA and c9,t11-CLA, respectively. The strongest effect of t9,t11-CLA was also observed in other colon cancer cell lines (HT-29 and DLD-1). The order of the inhibitory effect of CLA isomer was confirmed in the presence of 1% FBS. CLA isomers supplemented in the culture medium were readily incorporated into the cellular lipids of Caco-2 and changed their fatty acid composition. The CLA contents in cellular lipids were 26.2±2.7% for t9,t11-CLA, 35.9±0.3% for c9,t11-CLA and 46.3±0.8% for t10,c12-CLA, respectively. DNA fragmentation was clearly recognized in Caco-2 cells treated with t9,t11-CLA. This apoptotic effect of t9,t11-CLA was dose- and time-dependent. DNA fragmentation was also induced by 9c,11t-CLA and t10,c12-CLA. However, fragmentation levels with both isomers were much lower than that with t9,t11-CLA. t9t11-CLA treatment of Caco-2 cells decreased Bcl-2 levels in association with apoptosis, whereas Bax levels remained unchanged. These results suggest that decreased expression of Bcl-2 by t9t11-CLA might increase the sensitivity of cells to lipid peroxidation and to programmed cell death, apoptosis.

Keywords: Conjugated linoleic acid; Isomer specificity; Colon cancer cell; Apoptosis

1. Introduction

Conjugated linoleic acid (CLA) is a general term for the geometrical and positional isomers of octadecadienoic (18:2) acid with a conjugated double bond system. CLA is a naturally occurring substance in food sources, such as milk fat and the meat of ruminant animals. It occurs as mixtures of positional (ranging from 7,9- to 13,15-CLA) and geometrical [*cis*(c),c; c,*trans*(t); t,c; t,t] isomers and the major isomer is c9,t11-CLA [1]. A biological important role of CLA originated from beef was first reported as an anticarcinogen by Pariza and Hargraves [2]. It was later found that synthetic CLA had several beneficial effects in animal models, such as anticarcinogenesis [3,4], antiatherogene [5,6], decrease of body fat [7,8] and regulation of immunological reactions [9,10].

Synthetic CLA predominantly consists of c9,t11- and t10,c12-isomers. Although both isomers are known to possess biological activities, there is evidence indicating that more than one biological activity is involved in the specific effects of t10,c12-CLA. Data from animal models revealed that t10,c12-CLA reduces body fat and enhances lean body mass in mice [11]. Choi et al. [12] reported that t10,c12-CLA down-regulated stearoyl-CoA desaturase gene expression in 3T3-L1 adipocytes, while c9,t11-CLA did not alter adipocyte gene expression. Furthermore, Yamasaki et al. [13] reported that t10,c12-CLA but not c9,t11-CLA showed a potent cytotoxic effect on the rat hepatoma cells by activating the apoptotic pathway. The higher anticarcinogenic effect of

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t10,c12-CLA than c9,t11-CLA has been also found in breast cancer cells [14] and in Min mouse model [15].

The isomer-specific effects of c9,t11- and t10,c12-CLA suggest the different biological activity of each CLA isomer. However, there has been no report on the physiological effects of other CLA isomers such as t,t-isomers. In this study, we compared the inhibitory effect of four kinds of CLA isomers (c9,c11; c9,t11; t9,t11; t10,c12) on the growth of human colon cancer cells and found the stronger effect of t9,t11-CLA than the other two common CLA isomers.

2. Methods and materials

2.1. Materials

Colon cancer cells line, Caco-2 (HTB-37), HT-29 (HTB-38) and DLD-1 (CCL-221) were obtained from the American Type Culture Collection. c9,c11-CLA, c9,t11-CLA, t9,t11-CLA and t10,c12-CLA were purchased from Matreya (State College, PA, USA). Each CLA was converted to corresponding methyl ester with H_2SO_4-methanol solution [16]. Each methyl ester was analyzed by capillary gas chromatography (GC). GC analysis showed that the purity of CLA used was more than 99%. GC analysis was done on a Shimadzu GC-14B equipped with a flame-ionization detector and a capillary column [SP-2560 (100 m×0.32 mm i.d.); Supelco, Bellefonte, PA, USA]. The column temperature was held at 150°C for 47 min, raised to 200°C at the rate of 1°C/min then to 215°C at the rate of 5°C/min and finally held at 215°C for 20 min. Helium gas was used as the carrier at a flow rate of 1.2 ml/min. The injector and detector were held at 250°C and 260°C, respectively.

2.2. Cell culture conditions

Caco-2 cells were cultured in minimum essential medium supplemented with 10% or 1% fetal bovine serum (FBS), 1% nonessential amino acid, 100 U/ml penicillin and 100 μg/ml streptomycin. HT-29 and DLD-1 were cultured in Dulbecco's modified Eagle's medium and RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

2.3. Cell viability

Cell viability was assessed with 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt (WST-1, Wako Pure Chemical, Osaka, Japan), which is based on cleavage of the WST-1 reagent by mitochondrial dehydrogenase of viable cells to formazan dye [17]. Human colon cancer cells preincubated as described above were seeded at a density of 2×10^3 cells/well in 96-well microplates and cultured in 100 μL medium/well for 24 h. Each CLA was dissolved in 10 μl of 5% ethanol solution and then added to the culture. After 21 h of incubation, 10 μl of WST-1 solution was added to each well, and then the plate was incubated for a further 3 h. Cell viability was then measured spectrophotometrically at 450 nm (Microplate reader, Emax; Molecular Devices, Sunnyvale, CA, USA) and was expressed as a percentage of the viability obtained in control cultures, which were incubated 10 μl of 5% ethanol solution without the addition of CLA.

2.4. Measurement of DNA fragmentation

Quantitative measurement of apoptotic cells was performed using a commercial kit (Cell Death Detection ELISA^{PLUS}, 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. Cell culture conditions were the same as in cell proliferation assay.

2.5. Western blot analysis

Western blot analysis was carried out according a previous study [18]. Caco-2 cells (1.5×10^6 cells) were cultivated in 150-mm tissue cultured dish for 24 h, and t9,t11-CLA was then added into culture medium as ethanol solution. The final ethanol solution was below 0.1% (v/v). After incubation for 96 h, adherent cells were trypsinized and washed three times with phosphate buffered saline (PBS). Pellet was then scraped in a cold RIPA buffer (pH 7.4) containing 20 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mg/ml phenylmethylsulfonyl fluoride, 50 μg/ml apritinin and 1 mM Na_3VO_4. Further, cell lysates were centrifuged at 4°C, 15,000 rpm for 20 min. The supernatants (40 μg protein/lane) were separated by 10% SDS-polyacrylamide gel electophoresis. Proteins were transferred to polyvinylidene difluoride membrane, and membrane was then blocked with TBS-T] 20 mM Tris-HCl

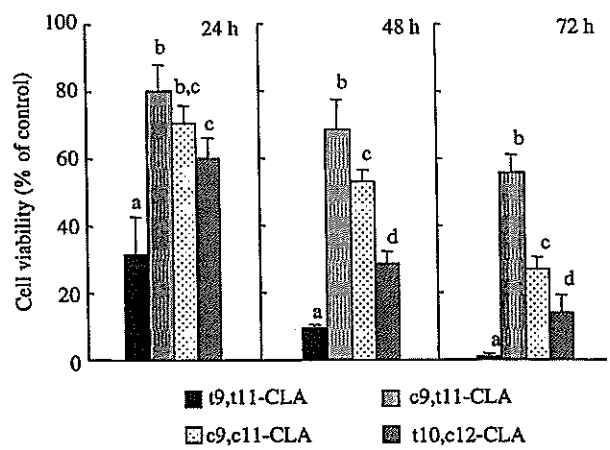

Fig. 1. Cell viability for Caco-2 cells incubated with four kinds of CLA (200 μM) up to 72 h. Data represent cell viability expressed as a percentage of the control, which was taken to be 100%. Incubation was carried out in the presence of 10% FBS. Data were means±S.D. for three samples. Values with different roman letters are significantly different ($P<.01$) at the same incubation time.

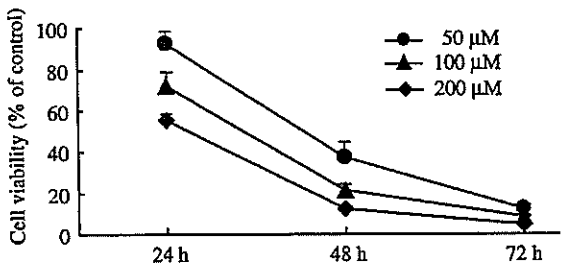

Fig. 2. Dose- and time dependent effect of t9,t11-CLA on the growth of Caco-2 cells. Incubation was carried out in the presence of 10% FBS. Data represent cell viability expressed as a percentage of the control, which was taken to be 100%. Data were means±S.D. for three samples.

(pH 7.6), 137 mM NaCl and 0.1% Tween 20] containing 5% nonfat dry milk for 1 h at room temperature. The membrane was incubated with anti Bcl-2 (Trevigen, Gaithersburg, MD, USA) and anti Bax (Trevigen) for 1 h. After washing, the membranes were incubated with a secondary antibody, anti-mouse IgG-HRP (Santa Cruz Biochemistry, Santa Cruz, CA, USA) for 1 h at room temperature. Finally, the membrane was treated with the reagents in the chemiluminescence detection kit (ECL system, Amersham Pharmacia Biotech, NJ, USA) according to the manufacturer's instructions. Actin was used as the control with human actin antibody (Santa Cruz Biotechnology). Densitometric analysis of the protein bands was performed with the software Scion Image (NIH Image, Bethesda, MD, USA).

2.6. Lipid extraction and analysis of the fatty acid composition

Caco-2 cells were preincubated as described above in 30 ml medium/dish for 48 h. Each CLA in ethanol was added to the culture at concentration of 50 μM. After 24 h of incubation, cells were washed twice with PBS and total lipids (TL) were extracted with chloroform/methanol (2:1, v/v). TL was transmethylated with 0.5 M CH_3ONa in MeOH by

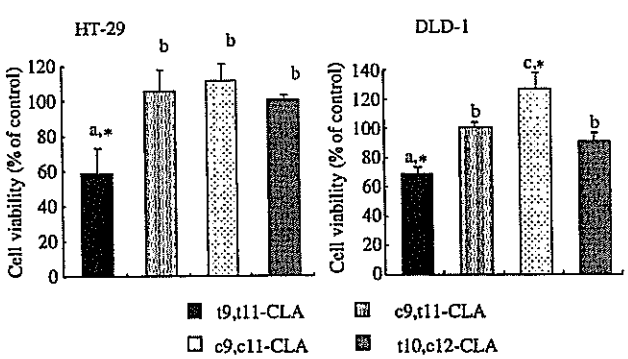

Fig. 3. Cell viability for DLD and HT cells incubated with four kinds of CLA (100 μM) for 72 h. Incubation was carried out in the presence of 10% FBS. Data represent cell viability expressed as a percentage of the control, which was taken to be 100%. Data were means±S.D. for three samples. Values with different roman letters are significantly different ($P<.01$). Values with asterisk mark denote significant difference from the control ($P<.01$).

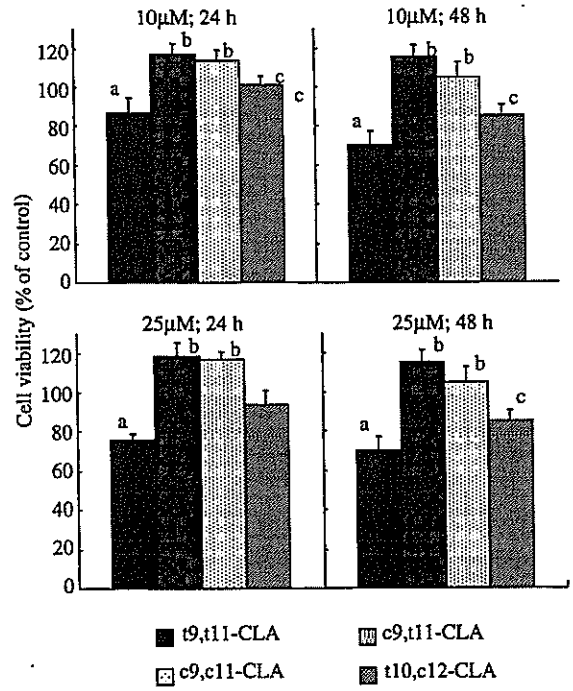

Fig. 4. Cell viability for Caco-2 cells incubated with four kinds of CLA (10 and 25 μM) up to 48 h. Data represent cell viability expressed as a percentage of the control, which was taken to be 100%. Incubation was carried out in the presence of 1% FBS. Data were means±S.D. for three samples. Values with different roman letters are significantly different ($P<.01$) at the same incubation time.

heating in a sealed tube at 60–70°C for 30 min under nitrogen. The fatty acid methyl esters were extracted with hexane. The extract was washed with water, dried over anhydrous sodium sulfate, concentrated in vacuo, purified by silicic acid column chromatography and then put through to GC. A capillary column [SP-2560 (100 m×0.32 mm i.d.); Supelco] was used for the identification of each CLA isomer

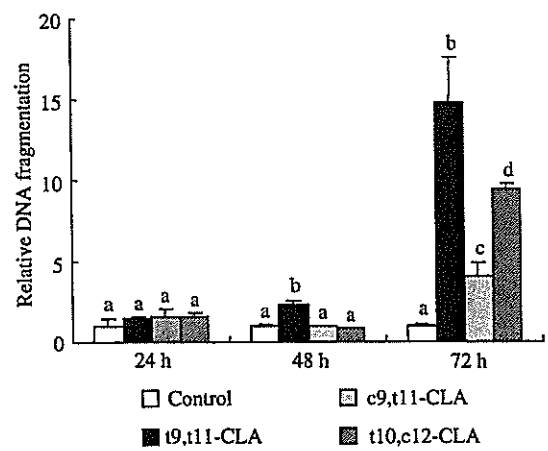

Fig. 5. DNA fragmentation in Caco-2 cells treated with t9,t11-, c9,t11- and t10,c12-CLA (200 μM). Incubation was carried out in the presence of 10% FBS. Relative DNA fragmentation was assigned the control to a value of 1.0. Data were means±S.D. for three samples. Values with different roman letters are significantly different ($P<.01$) at the same incubation time.

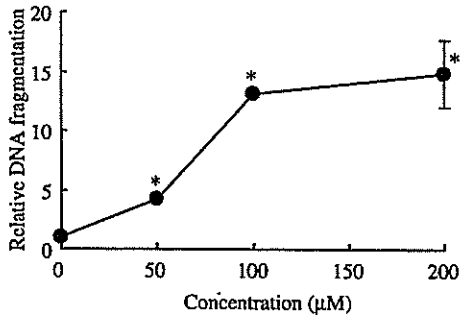

Fig. 6. Dose-dependent apoptotic effect of t9,t11-CLA on Caco-2 cells. Caco-2 cells were incubated with different concentrations of each CLA for 72 h. Incubation was carried out in the presence of 10% FBS. Relative DNA fragmentation was assigned the control (0 μM of t9,t11-CLA) to a value of 1.0. Data were means±S.D. for three samples. Values with asterisk mark denote significant difference from the control ($P<.01$).

in the methyl esters. The GC condition was the same as described above. Fatty acid profile was analyzed by a Shimadzu GC-14B equipped with a flame-ionization detector and a capillary column [Omegawax 320 (30 m×0.32 mm i.d.); Supelco]. The column temperature was held at 200°C. The injection port and flame ionization detector were operated at 250°C and 260°C, respectively. Helium was used as carrier gas, and its flow was 4.1 ml/min. The fatty acid methyl esters were identified by comparison of retention times with authentic standards and with the equivalent chain length (ECL) values. The analysis was triplicate and there was little difference in the oxygen consumption and polymer formation rates for each determination.

The analysis was done three times for the same sample and calculated ECL values are the mean values of several GC runs.

2.7. Statistical analysis

Data are expressed as means±S.D. Statistical analyses between multiple groups were determined by analysis of variance. Statistical comparisons were made by Scheffe's F test.

3. Results

When Caco-2 cells were incubated with 200 μM CLA isomer each for 24 h, all isomers caused a reduction in cell viability (Fig. 1). This reduction occurred in a time-dependent manner in all isomers. Among them, t9,t11-CLA showed the strongest inhibitory effect on the cell growth during the whole culture period, followed by c10,t12-CLA, c9,t11-CLA and c9,t11-CLA, respectively. The strong activity of t9,t11-CLA was confirmed by dose- and time-dependent growth inhibition on Caco-2 cells (Fig. 2). The same tendency was observed in other kinds of colon cancer cells. As shown in Fig. 3, t9,t11-CLA (100 μM) significantly reduced the viability of DLD-1 and HT-29 colon cancer cells as compared with the control, while other CLA isomers showed no inhibitory effect on both colon cancer cells at 100 μM. Fatty acid can form complex with bovine serum albumin. In the present experimental conditions, a part of CLA would form complex with the albumin in the medium. Fig. 4 shows the effect of CLA on Caco-2 cell viability in 1% FBS concentration. In the presence of 10% FBS, any CLA had no inhibitory effect on cell growth under 25 μM within 48 h incubation. On the other hand, t9,t11-CLA and t10,c12-CLA significantly inhibited the cell growth at 10 and 25 μM by the reduction of FBS to 1%. The effect of t9,t11-CLA was higher than that of t10,t12-CLA.

To characterize the mechanism of cell death by CLA, cytological alterations and DNA degradation of Caco-2 cells were analyzed. Caco-2 cells killed by t9,t11-CLA were accompanied by nuclear condensation, and brightness occurred as observed by fluorescent microscope of the stained cells. The measurement of cytoplasmic histone-associated DNA fragments as an indicator of apoptosis showed that t9,t11-CLA (200 μM) significantly induced DNA fragmentation in Caco-2 cells after 48 h of incubation (Fig. 5). However, there were no significant effects of c9,t11-CLA and t10,c12-CLA (200 μM) on DNA fragmentation after 48 h of incubation. The DNA fragmentation by c9,t11-CLA and t10,c12-CLA was found after 72 h of incubation, but the levels were lower than that by t9,t11-CLA. Fig. 6

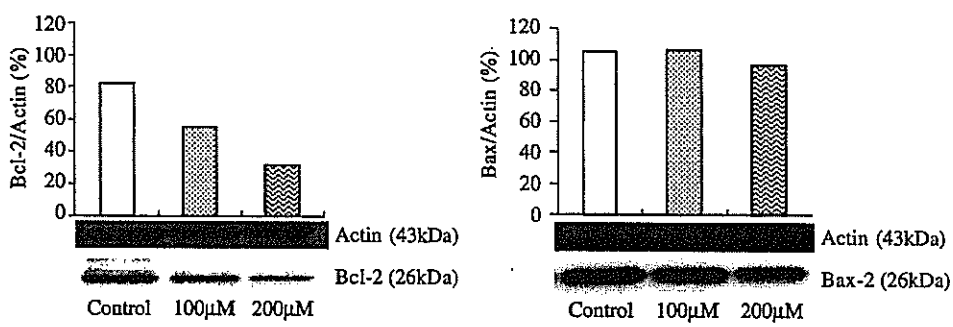

Fig. 7. Effect of t9,t11-CLA treatment on Bcl-2 (A) and Bax (B) protein levels in Caco-2 cells. Incubation was carried out in the presence of 10% FBS. Reactive protein bands were analyzed using chemiluminescence detection kit (ECL system) with specific antibodies. Expressed proteins were represented as the percentage to the control β-actin. Each bar represents the approximate amount of protein corresponding to the banding pattern that was measured by densitometric analysis performed with the software Scion Image (NIH Image).

Table 1
Incorporation of CLA into the cellular total lipids after incubation for 24h[a]

Fatty acid (wt. %)	Cell			
	Control	+t9,t11-CLA	+c9,t11-CLA	+t10,c12-CLA
14:0	2.5±0.2	2.1±0.5	1.7±0.1	1.2±0.0
16:0	22.2±0.2	13.4±2.1	15.0±0.5	10.8±0.0
18:0	7.6±0.0	4.0±0.1	5.2±0.2	5.5±0.1
16:1n-7	8.2±0.5	6.5±1.3	4.3±0.1	2.5±0.1
18:1n-7	10.8±0.0	6.6±0.2	6.4±0.1	4.8±0.0
18:1n-9	24.2±0.1	18.3±0.8	14.2±0.3	10.7±0.0
18:2n-6	3.1±0.0	2.6±0.1	2.0±0.1	1.9±0.0
20:4n-6	8.6±0.4	6.6±0.1	5.7±0.1	5.6±0.1
22:6n-3	2.6±0.5	1.9±0.7	1.9±0.0	1.9±0.1
t9,t11-CLA	ND	26.2±2.7	ND	ND
c9,t11-CLA	ND	ND	35.9±0.3	ND
t12,c12-CLA	ND	ND	ND	46.3±0.8

ND, not detected.
[a] Data are expressed as mean±SD (n=3).

shows the DNA fragmentation in Caco-2 cells incubated with different concentrations of t9,t11-CLA for 72 h. Significant induction of DNA fragmentation was observed at 50 μM, and this effect was in a dose-dependent manner.

In an attempt to explore the effects of t9,t11-CLA on apoptosis-regulating proteins, we examined expression of Bcl-2 and Bcl-xL, which suppress programmed cell death, and that of Bax, which appears to promote it. Fig. 7 shows the results of immunohistochemical analysis of Bc1-2 expression (Fig. 7A) in Caco-2 cells incubated with varying t9,t11-CLA concentrations (0, 100 and 200 μM) for 96 h. Treatment with the CLA reduced the percentage of Bcl-2 protein expression in a dose-dependent manner. In contrast, no changes in the percentage of Bax expression (Fig. 7B) were found following a 96-h t9,t11-CLA treatment.

When Caco-2 cells were incubated with CLA for 24 h, significant amount of each isomer was incorporated into the cancer cells. On the other hand, these CLAs were not detected in control cells (Table 1). Main fatty acid of control cells were 18:1n-9, 16:0, 18:1n-7, 20:4n-6, 16:1n-7 and 18:0. The levels of these fatty acids decreased with the incorporation of CLA into the cells. Furthermore, two peaks (<1%) were detected between peaks of 20:4n-3 and 22:6n-3, which were not observed in the methyl esters from TL of control cells. Judging from ECL values, both peaks are suggested to be elongated metabolites of CLA isomers. Moreover, a new peak appeared between peaks of 16:1n-7 and 18:1n-9 in the methyl esters from TL of CLA-supplemented cells. The contents of this new peak were 3.3±0.2% for t9,t11-CLA, 0.5±0.1% for c9,t11-CLA, and 1.7±0.0% for t10,c12-CLA. Judging from the ECL of each peak and absence of this peak in control cells, these peaks would be derived from each CLA isomer with less degree of unsaturation.

4. Discussion

It has been considered that both c9,t11-CLA, which is the CLA isomer present in highest amounts in milk fat, as well as t10,c12-CLA, which is present at considerable lower levels naturally, are equipotent in terms of their ability to inhibit mammary carcinogenesis. However, recent in vitro studies [13,19,20] showed that t10,c12-CLA is somewhat more efficacious than c9,t11-CLA for many parameters, suggesting the possibility that lower doses of t10,c12-CLA can be utilized in vivo, with good efficacy and low toxicity. The results in Fig. 1 confirmed the higher activity of t10,c12-CLA than c9,t11-CLA.

There have been many papers on the anticarcinogenic activity of c9,t11-CLA and t10,c12-CLA. However, the effect of other naturally occurring CLA isomer has not been investigated. In the present study, inhibitory effect of t9,t11-CLA on the growth of colon cancer cells was compared with those of c9,c11-CLA, c9,t11-CLA and t10,c12-CLA. As shown in Figs. 1, 3 and 4, t9,t11-CLA showed the strongest cytotoxic effect on colon cancer cells. The high activity of t9,t11-CLA was consistent with the level of apoptosis induced by this CLA isomer in Caco-2 cells (Fig. 5). In apoptotic pathway, the death signal triggers a cascade of various molecular actions resulting in the cleavage of genomic DNA and specific proteins. It has been reported that CLA induces apoptosis in cancer cells such as mammary adenocarcinoma [21], normal rat mammary epithelical cells [22], and human hepatoma [23]. However, the data on isomer specificity has been limited [13]. The present study demonstrated the higher inhibitory effect of t9,t11-CLA on the growth of human colon cancer cells with apoptotic pathway than those of t10,c12-CLA, c9,t11-CLA and c9,c11-CLA.

In some studies on the inhibitory effect of CLA on the cancer cells, cells were cultured in a medium without FBS [22–25]. Yamasaki et al. [13] reported that CLA at 10 μM did not affect the cell growth in the presence of 5 or 10% FBS. To obtain the clear result on the inhibitory effect of CLA, FBS concentration might reduce less than 1%. On the other hand, Chujo et al. [20] reported that FBS contains growth factors that induce proliferation of human breast cancer cells. Two percent of FBS was used in the study on the effect of CLA using adipose cells [12] and hepatic cells [26]. Changes in gene expression of human cancer cells by docosahexaenoic acid was determined in cell culture medium containing 5% and 10% FBS [27–29]. When docosahexaenoic acid was added to MCF-7, MDA-MB-231 and SiHa cells and incubated in the medium containing 5% FBS, the inhibition of cell growth was found at more than 300 μM docosahexaenoic acids. In the present study, inhibitory effect of different CLA isomers on cell growth was compared in culture medium containing 10% FBS (Figs. 1 and 2) and 1% FBS (Fig. 1). In both cases, the strongest inhibitory effect on the growth of caco-2 cells was shown by t9,t11-CLA, followed by t10,c12-CLA, c9,c11-CLA and c9,t11-CLA. Although concentration of FBS (10%) and CLA (100 or 300 μM) used in the present study was far away from physiological condition, the result obtained in the study will

provide important information of chemopreventive effect of CLA isomer.

CLA mixture (predominantly c9,t11-CLA and t10,c12-CLA) is obtained by the chemical isomerization of linoleic acid. Industrial separation of t10,c12-CLA can be done by chemical procedure from this CLA mixture. On the other hand, t9,t11-CLA is a minor component in the CLA mixture, and a large-scale separation of t9,t11-isomer would be impossible. t9,t11-CLA is known to be contained in milk fat and dairy products. In this case, t9,t11-CLA would be biologically synthesized. Kishino et al. reported [30,31] the bacterium production of two CLA isomers (c9,t11- and t9,t11-CLA) from ricinoleic acid [30] and linoleic acid [31]. Among both isomers, t9,t11-CLA was the main component, and this isomer was produced at more than 97% purity by lactic acid bacteria if the reaction is done long enough with a low linoleic acid concentration [32]. This biological system for t9,t11-CLA production may promise the large-scale and selective preparation of t9,t11-CLA.

Agatha et al. [33] reported that c9,t11-CLA and c9,c11-CLA supplemented in the culture medium were readily incorporated and esterified into a human leukemia cellular phospholipid in a concentration- and time-dependent manner. The cellular phospholipids contained high level of CLA (range: 32-63 g/100 g total phospholipids). As shown in Table 1, CLA was also easily incorporated into human colon cancer cells (Caco-2) leading to an extensive alteration of the fatty acid profile of the cellular lipids. In the human leukemia cells, the supplementation with CLA resulted in the accumulation of the corresponding fatty acid with conjugated double bonds by desaturation/elongation system [33]. However, a little amount of metabolites of CLA with long-chain and/or higher degree of unsaturation was detected in the total lipids of human colon cancer cells (Caco-2) supplemented with CLA (200 μM) for 72 h of incubation.

The mechanism of action of CLA is still unknown. However, different mechanisms of the anticarcinogenic activity of CLA have been hypothesized. Studies show that the growth inhibitory effect of CLA on cancer cell lines is related to the increase in lipid peroxidation, alteration of cellular fatty acid composition and regulation of some gene expressions [34]. The present study showed the changes in the fatty acid composition of cellular lipid by each CLA supplementation (Table 1). However, the change in fatty acid composition by t9,t11-CLA, which showed the most potent inhibitory effect on colon cancer cells (Figs. 1, 3 and 4), was relatively smaller than those by t10,c12-CLA and c9,t11-CLA (Table 1), suggesting no relation of the activity of t9,t11-CLA with the change in fatty acid composition. It has been reported that overexpression of Bcl-2 in transgenic models leads to protection of many cell types against apoptosis induced by a variety of oxidative stresses, including lipid peroxidation, suggesting that Bcl-2 exerts an antioxidative function [18,35–37]. In addition, anti-apoptotic activity of Bcl-2 is found to be antagonized by a homologous Bax protein, which is able to form heterodimers with Bcl-2. Thus, the ratio of Bax to Bcl-2 within the cell is the critical determining factor for the propensity of a cell to undergo apoptosis. In this study, we showed that t9,t11-CLA treatment led to a decrease in Bcl-2 expression but not the level of Bax. The decreased Bcl-2 levels along with normal levels of Bax may be sufficient to shift the balance toward apoptosis in Caco-2 cells.

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Diet supplemented with citrus unshiu segment membrane suppresses chemically induced colonic preneoplastic lesions and fatty liver in male *db/db* mice

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The modulatory effects of dietary citrus unshiu segment membrane (CUSM) on the occurrence of aberrant crypt foci (ACF) and β-catenin accumulated crypts (BCACs) were determined in male C57BL/KsJ-*db/db* (*db/db*) mice initiated with azoxymethane (AOM). Male *db/db*, *db*/+ and +/+ mice were given 5 weekly subcutaneous injections of AOM (15 mg/kg body weight), and then they were fed the diet containing 0.02%, 0.1% or 0.5% CUSM for 7 weeks. At Week 12, a significant increase in the numbers of ACF and BCAC was noted in the *db/db* mice in comparison with the *db*/+ and +/+ mice. Feeding with CUSM caused reduction in the frequency of ACF in all genotypes of mice and the potency was high in order of the *db/db* mice, *db*/+ mice and +/+ mice. The number of BCACs was also reduced by feeding with CUSM, thus resulting in a 28–61% reduction in the *db/db* mice, possibly due to suppression of cell proliferation activity in the lesions by feeding with CUSM-containing diet. Clinical chemistry revealed a low serum level of triglyceride in mice fed CUSM. In addition, CUSM feeding inhibited fatty metamorphosis and fibrosis in the liver of *db/db* mice. Our findings show that CUSM in the diet has a chemopreventive ability against the early phase of AOM-induced colon carcinogenesis in the *db/db* as well as *db*/+ and +/+ mice, indicating potential use of CUSM in cancer chemoprevention in obese people.
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Key words: citrus unshiu segment membrane; ACF; BCAC; colon carcinogenesis; *db/db* mice

The modern Western lifestyle, including a high caloric intake, high-fat diets and physical inactivity, results in a positive energy balance, diabetes and obesity. These lifestyle patterns might also be risk factors for the development of colorectal cancer (CRC),[1] which is one of the major causes of morbidity and mortality in the Western world.[2] This malignancy has also increased in Asia owing to the changes in lifestyle, such as the dietary habit of increased meat consumption.[2,3] Several prospective and case-control studies have addressed the relationship between obesity/diabetes and CRC.[1,4,5]

C57BL/KsJ-*db/db* (*db/db*) mice are used as a genetically altered animal model with genotypes of obesity and diabetes mellitus.[6] A disruption of the leptin receptor (Ob-R) gene in these mice leads to an over-expression of leptin in the adipose tissue and a concomitantly high serum concentration of leptin.[7,8] The synthesis of leptin in adipocytes is influenced by insulin,[9] tumor necrosis factor-α,[10] glucocorticoids,[11] reproductive hormones[12] and prostaglandins[13] that may be involved in the neoplastic processes.[14] In addition, leptin can act as a growth factor in colonic epithelial cells[15] while modulating the proliferation of colonic cryptal cells.[16] In contrast, more leptin in the blood clearly decreased colon carcinogenesis in 3 different animal models.[17,18] Thus, leptin might be one of the biological factors involved in the development of CRC associated with obesity/diabetes. The *db/db* mouse, therefore, is a very useful model for elucidating the relationship between colon carcinogenesis and obesity/diabetes.

Certain food components are known to exert a cancer chemopreventive activity against CRC development.[19] However, few studies have so far been performed on the preventive effect of food components on obesity/diabetes-related colon carcinogenesis.[20,21] We recently have made the citrus unshiu segment membranes (CUSMs) that are rich in soluble and insoluble fiber and separate the juice vesiculates, from Satsuma mandarin (Citrus unshiu Marc.). Mandarin orange fruit constitutes 9–13 segments (juice sacs) that contain juice vesicles, and a membrane that wraps the segment is called "segment membrane." Although CUSM is waste product that remains after squeezing citrus unshiu for fruit juice, it contains biologically active compounds such as flavonoids, including hesperidin. Citrus fibers and flavonoids have been reported to inhibit colon carcinogenesis in rodents.[22–24] Obese individuals are thus often recommended to consume such diet low-energy foods rich in fiber with a possibly specific hypolipidemic effect, such as pectin-enriched dishes, fruit purees and juices and wheat bran bisquits.[25] Supplementation with flavonoids (hesperidin or naringin) improves the hyperglycemia in *db/db* mice.[26] In addition, CUSMs possess an antiobesity effect *in vitro* (Suzuki *et al.*, unpublished work). Although the biological activity of CUSM has yet to be elucidated, we suspected that CUSM might have a preventive effect on obesity/diabetes-related colon carcinogenesis.

In the current study, we determined the possible modulatory effects of dietary CUSM on the occurrence of azoxymethane (AOM)-induced aberrant crypt foci (ACF) and β-catenin accumulated crypts (BCACs), which are putative precursor lesions for colonic adenocarcinoma,[27,28] in *db/db*, *db*/+ and +/+ male mice. Since we previously observed the immunohistochemical overexpression of Ob-R and insulin-like growth factor-I receptor (IGF-1R) in AOM-induced BCACs in *db/db* mice,[29] the effects of CUSM on the expression of Ob-R and IGF-1R in BCACs and their surrounding cryptal cells were also investigated. Also, the effect of CUSM feeding on the cell proliferating activity of BCACs was assessed by counting proliferating cell nuclear antigen (PCNA)-index in the lesion.

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; BCACs, β-catenin accumulated crypts; CRC, colorectal cancer; CUSM, citrus unshiu segment membrane; IGF-1R, insulin-like growth factor-I receptor; H & E, hematoxylin and eosin; NF-κB, nuclear factor kappa B; Ob-R, leptin receptor; PCNA, proliferating cell nuclear antigen.

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