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[1]	ADC[2]	Total	AD	ADC
1	AOM + 1% DSS	10	10/10 (100)[3]	10/10 (100)	10/10 (100)	5.40 ± 1.71[4]	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)[5]	3.10 ± 2.28	2.10 ± 1.79	1.00 ± 1.33[6]
3	AOM + 1% DSS/0.05% auraptene	10	6/10 (60)[7]	6/10 (60)[7]	4/10 (40)[8]	1.70 ± 1.70[9]	1.10 ± 1.29	0.60 ± 0.84[9]
4	AOM + 1% DSS/0.01% collinin	10	7/10 (70)	6/10 (60)[7]	4/10 (40)[8]	2.90 ± 2.33	2.00 ± 1.83	0.90 ± 1.20[6]
5	AOM + 1% DSS/0.05% collinin	5	6/10 (60)[7]	5/10 (50)[5]	4/10 (40)[8]	1.40 ± 1.43[9]	0.80 ± 0.92	0.60 ± 0.84[9]
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

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

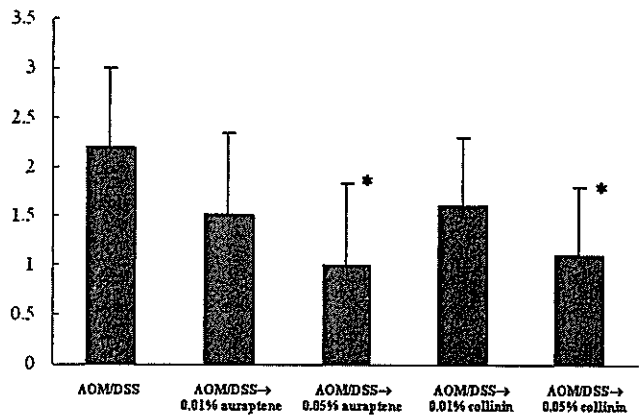

FIGURE 4 – Inflammation score. Statistical analysis using Bonferroni multiple comparison post test indicates significant difference (*$p < 0.05$), *vs.* the AOM/DSS group.

and collinin on the development of colonic adenocarcinoma was well correlated with the inhibition of cell proliferation activity, induction of apoptosis and inhibition of imumunoreactivity 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,[36] 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.[37,38] 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.[39] In neoplastic tissues, changes in cell proliferation and apoptosis are regarded as a common denominator in the pathogenesis of tumor formation.[40] 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 promotion stage of cancer development.[41] 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.[42,43] 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_2O_2 production and cell proliferation.[44] In addition, auraptene quite likely reduces the production of lipid peroxidation products in rat colon carcinogenesis.[24] 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,[29,30] 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.[45,46] We also observed increased expression of COX-2 and iNOS in colon adenocarcinomas in this animal model.[28] The increases in the reaction products of iNOS and COX-2, nitric oxide and PGE_2 respectively, could contribute to colon tumorigenesis. Expression and activity of iNOS are increased in the colonic mucosa in patients with IBD[47] and colonic adenomas.[48] 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.[26,49] An iNOS-selective inhibitor could suppress the development of AOM-induced colonic preneoplastic lesions by inhibition of iNOS activity.[50] Likewise, an increased COX-2 expression is reported in human and rodent CRC,[51,52] and its overexpression may confer a survival advantage on cells by inhibition apoptosis and a change in cellular adhesion to the extracellular matrix.[53] 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.[54] Our previous study[55] and those of others[56,57] shows that COX-2 inhibitors inhibited colon tumorigenesis as well as colitis, induced by naturally occurring carcinogen. Suh *et al.*[58] synthesized novel synthetic triterpenoids that suppressed iNOS and COX-2 protein expression, and demonstrated their potent differentiating, antiproliferating and anti-inflammatory activities.[59] 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[1](20)[2]	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[3](10)	18.1 ± 5.0[4](10)	2.4 ± 1.2[3](10)	2.3 ± 0.8[5](10)	1.7 ± 0.8(10)
3	AOM + 1% DSS/0.05% auraptene	47.2 ± 13.4[3](6)	20.7 ± 5.4(6)	2.0 ± 0.9[4](6)	1.8 ± 1.0[5](6)	1.4 ± 0.7[5](6)
4	AOM + 1% DSS/0.01% collinin	51.8 ± 10.0[3](9)	19.1 ± 5.6[4](9)	2.6 ± 1.0(9)	2.4 ± 0.7[3](9)	1.8 ± 0.8(9)
5	AOM + 1% DSS/0.05% collinin	49.3 ± 13.2[4](6)	21.3 ± 6.9[3](6)	2.3 ± 1.2[4](6)	2.2 ± 1.3[3](6)	1.3 ± 0.5[4](6)

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

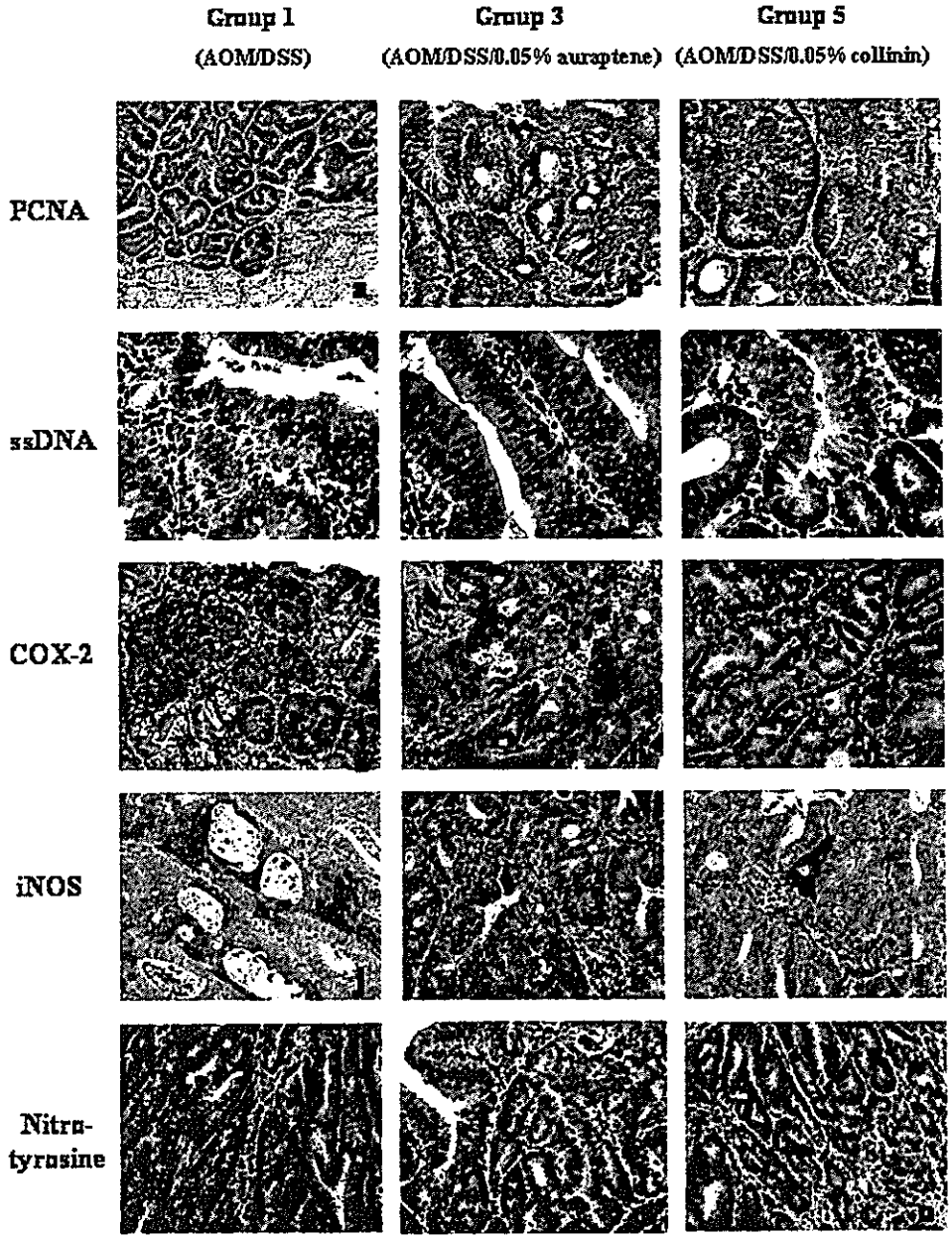

FIGURE 5 – Immunohistochemistry of PCNA, ssDNA, COX-2, iNOS and nitrotyrosine in adenocarcinomas. When compared to group 1 (*a*), the numbers of PCNA-positive nuclei in adenocarcinomas developed in mice from groups 3 (*b*) and 5 (*c*) were low. In contrast to ssDNA positivity (*d*) in adenocarcinoma 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 (*n*) 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*) nitrotyosine 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-α.[19] Our recent study[29] 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 carcinogenesis. 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)-γ (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 PPARγ protein in the non-tumorous colonic mucosa (12). Thus, proinflammatory genes and PPARγ 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

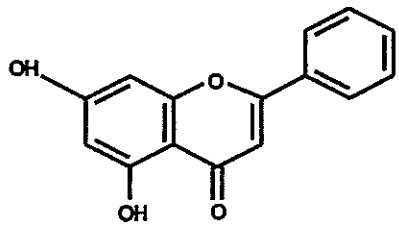

Figure 1. Chemical structure of chrysin.

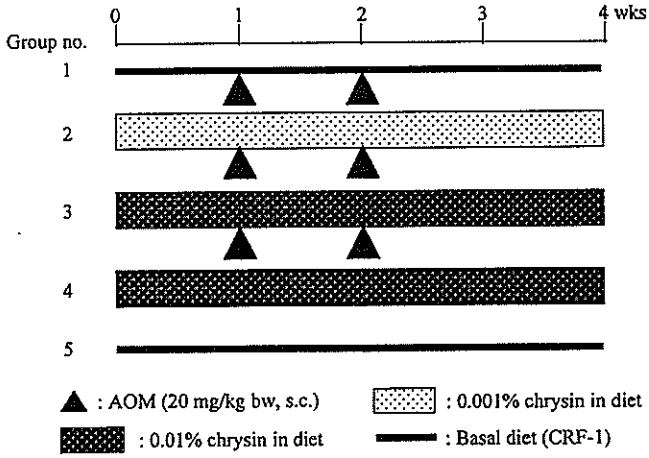

Figure 2. Experimental protocol.

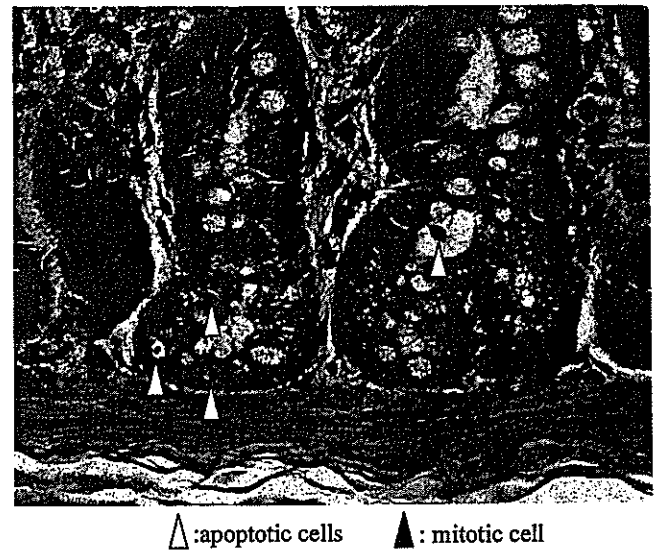

Figure 3. Apoptotic and mitotic cells in the crypt from the distal colon, which was stained with hematoxylin and eosin, from a rat in group 3 (AOM+0.01% chrysin). Apoptotic cells are identified by cell shrinkage, homogeneous basophilic and condensed nuclei, nuclear fragments (apoptotic bodies), marked 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.

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±10%), lighting (12-h light/dark cycle), and temperature (23±2°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 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 μm-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[a]	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

[a]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[a]	145±28	1.98±0.10
2	AOM+0.001% chrysin (8)	8/8 (100%)	37±17[b]	67±29[b]	1.81±0.14[c]
3	AOM+0.01% chrysin (8)	8/8 (100%)	40±10[b]	69±21[b]	1.73±0.09[b]
4	0.01% chrysin (4)	0/4 (0%)	0	0	0
5	No treatment (4)	0/4 (0%)	0	0	0

[a]Mean ± SD, [b]significantly different from group 1 by one-way ANOVA with Bonferroni correction (P<0.001), [c]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[a]	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[b]	0.3±4.1[b]	7.1±6.1
3	AOM+0.01% chrysin (8)	47.3±5.5	34.4±6.4	16.3±3.9[c]	2.0±3.1[b]

[a]Mean ± SD, [b]significantly different from group 1 by one-way ANOVA with Bonferroni correction (P<0.01), [c]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 Softwear, 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[a,b]	1.2±1.6	44.2±10.1[b]
2	AOM+0.001% chrysin (38)	3.2±2.5	3.2±2.3[c]	43.3±6.9
3	AOM+0.01% chrysin (57)	1.4±1.4[c]	3.7±2.1[c]	55.4±10.2[c]
4	0.01% chrysin (56)	1.8±1.4	1.2±1.2	54.0±11.1[d]
5	No treatment (28)	1.3±1.4	0.8±1.0	62.0±11.7

[a]Mean ± SD, [b]significantly different from group 5 by one-way ANOVA with Bonferroni correction (P<0.001), [c]significantly different from group 1 by one-way ANOVA with Bonferroni correction (P<0.001), [d]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^{-1} with AUCs of 5-193 ng ml^{-1} h, whereas chrysin sulphate concentrations were 30-fold higher (AUC 450-4220 ng ml^{-1} 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 artepillin 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.

Acknowledgements

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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

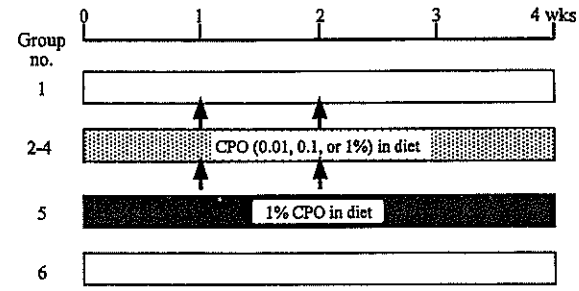

Figure 1. Experimental protocol. White bar, basal diet; dotted bar, CPO (0.01, 0.1, and 1%); dashed bar, CPO 1%; arrow, AOM, 20 mg/kg bw s.c.

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 (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,

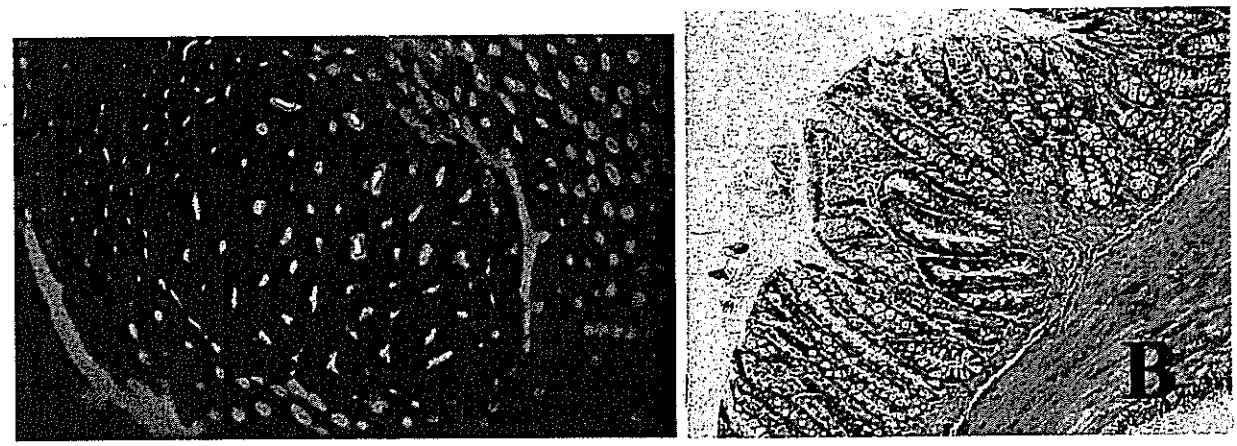

Figure 2. Morphology of representative ACF found in a rat from group 1. (A) ACF on methylene-blue-stained colonic mucosa; and (B) ACF on hematoxylin and eosin-stained section. Original magnification, (A) x4; and (B) x10.

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 ($P<0.001$).

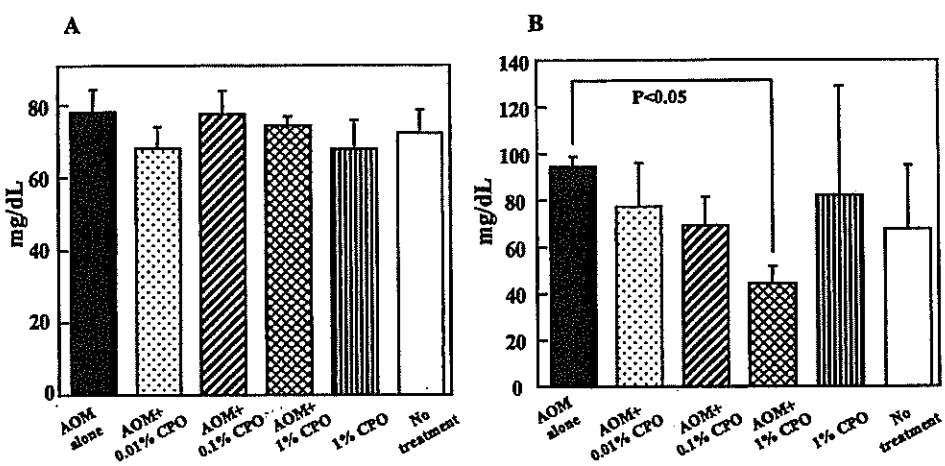

Figure 3. Effect of CPO on total cholesterol and triglyceride in serum. (A) Total cholesterol level, (B) triglyceride level.

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

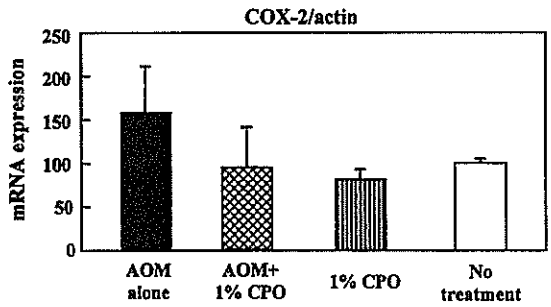

Figure 4. Effect of CPO diet on COX-2 mRNA expression in rat colon mucosa.

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,c13-18:3 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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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.

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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Material and methods

Animals, chemicals and diets

Four-week-old male *db/db* mice, *db/+* mice and +/+ mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). All mice were maintained at the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines, and were housed in polycarbonate cages (4–5 mice/cage) with free access to drinking water and a basal diet, MF (Oriental Yeast Co., Ltd., Tokyo, Japan), under controlled conditions of relative humidity [(50 ± 10)%], lighting (12-h light/dark cycle) and temperature [(23 ± 2)°C]. AOM was purchased from the Sigma Chemical Co. (St. Louis, MO). Powdered CUSM was obtained from the Ehime Beverage Inc. (Matsuyama, Japan). The composition of CUSM (100 g) was as follows: 2.4 g moisture; 5.5 g protein; 0.3 g fat; 51 g fiber (22.0 g soluble and 29.0 g insoluble); 2.3 g ash; 26.6 g saccharide (6.1 g D-flucutose, 5.5 g glucose and 15.0 g D-suclose); 2.2 g hesperidin and 9.7 g others that include flavonoids, carotenoids and unknown components. The experimental diets were prepared by mixing CUSM into the basal diet at a dose of 0.02%, 0.1% or 0.5% on a weekly basis.

Experimental procedures

Male homozygous *db/db* mice (36 mice), heterozygous *db/+* mice (40 mice) and littermate controls (+/+) mice (40 mice) were divided into 4 groups, respectively. At 5 weeks of age, all mice were subcutaneously injected with AOM (15 mg/kg body weight) once a week for 5 weeks. Group 1 was fed the basal diet throughout the experiment. Groups 2 through 4 were fed the diets containing CUSM at dose levels of 0.02%, 0.1% and 0.5%, respectively, for 7 weeks, starting one week after the last injection of AOM. The experiment was terminated 12 weeks after the start.

All mice were provided with the experimental diets and tap water *ad libitum*, and were weighed weekly. The food intake of the animals was monitored every day. At the termination of the study (Week 12), all mice were sacrificed by an overdose of ether to analyze the number of AFC and BCACs. At autopsy, all organs, including the intestine, were carefully examined grossly, and then were examined histopathologically. The weighed liver and kidney were also submitted for histological examinations to investigate the toxicity of CUSM.

Identification of ACF and BCACs

The presence of ACF and BCACs was determined according to the standard procedures that are routinely used in our laboratory.[30,31] 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 their mucosal surface up and then fixed in 10% buffered formalin for at least 24 hr. The fixed colons were stained with methylene blue (0.5% in distilled water) for 20 sec, dipped in distilled water and placed on a microscopic slide to count the ACF. After counting the ACF, the distal parts (1 cm from the anus) of the colon were cut in order to count the number of BCACs. To identify BCAC intramucosal lesions, the colon (0.58–0.87 cm^2/colon) was embedded in paraffin, and then a total of 20 serial sections (4-μm thick each) per mouse were made by an *en face* preparation.[32,33] For each case, 2 serial sections were used to analyze the BCACs.

Histopathology and immunohistochemistry

Five serial sections were made from paraffin-embedded blocks. Two sections were subjected to hematoxylin and eosin (H & E) staining for histopathology and β-catenin immunohistochemistry to count the number of colonic BCACs,[32,33] and others were used for Ob-R, IGF-1R and PCNA immunohistochemistry. Immunohistochemistry for β-catenin was performed on 4-μm-thick paraffin-embedded sections from the distal segments of the colons, using the labeled streptavidin-biotin method (LSAB KIT; DAKO, Glostrup, Denmark) with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65°C, deparaffinized in xylene and rehydrated through graded alcohols at room temperature. A 0.05 M Tris-HCl buffer (pH 7.6) was used to prepare solutions and for washes between various steps. The sections were treated for 40 min at room temperature with 2% bovine serum albumin and incubated overnight at 4°C with a primary antibody against β-catenin protein (diluted 1:1,000, Transduction Laboratories, Lexington, KY). Horseradish peroxidase activity was visualized by treatment with H_2O_2 and diaminobenzidine for 5 min. Negative control sections were immunostained without the primary antibody. Immunoreactivity was regarded as positive if apparent staining was detected in the cytoplasm and/or nuclei to determine the BCACs.

Immunohistochemistry of Ob-R and IGF-1R was performed using a stain system kit (Zymed, South San Francisco, CA). Rabbit polyclonal antibodies against Ob-R (1:200 dilution, sc-8325, Santa Cruz Biotechnology, Santa Cruz, CA) and IGF-1Rα (1:150 dilution, sc-7952, Santa Cruz Biotechnology) were applied overnight to the sections at 4°C according to the manufacturer's protocols. Human CRC samples were used as positive controls. The immunoreactivity cells were considered to be positive when definite cytoplasmic staining was identified. PCNA immunohistochemistry was performed on 4-μm-thick paraffin-embedded sections from colons of the *db/db* mice 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. Cells with intensively stained nuclei were considered to be positive for PCNA, and the indices (%) were calculated in each BCAC. Calculation was done in 20 BCACs from Group 9, 15 BCACs from Group 10, 10 BCACs from Group 11 and 8 BCACs from Group 12.

Morphometric analysis

Two serial sections from the liver of all mice were made for a morphometric analysis of liver fibrosis and fatty change. Liver sections were stained with H & E for histopathology and Sirius-red for morphometry of fibrosis. Fatty metamorphosis (% of fatty degeneration) was determined on the H & E-stained liver section, and liver fibrosis was expressed as the % of fibrosis in the area of liver section. An image analysis software, NIH Image v.1.63, was used for these calculates.

Clinical chemistry

At sacrifice, blood to measure the serum concentrations of glucose, leptin, insulin, cholesterol and triglyceride levels was collected from 5 mice, each of genotypes +/+, *db/+* and *db/db*. They were starved overnight prior to blood collection for clinical chemistry. The serum glucose level was measured enzymatically using the hexokinase method. The serum triglycerides were assayed by enzymatic hydrolysis with lipase. Serum cholesterol was determined enzymatically using cholesterol esterase and cholesterol oxidase. Serum concentrations of leptin and insulin were measured by an enzyme immunoassay according to the manufacturer's protocol (R & D systems, Minneapolis, MN).

Statistical evaluation

Where applicable, the data were analyzed using one-way ANOVA with Bonferroni correction or Fisher's exact probability test, with $p < 0.05$ as the criterion considered to indicate significance.

Results

General observations

The carefully monitored food intake of the animals showed that the mean daily intakes of *db/db* mice (7.26 g in the AOM alone group; 7.10 g in the AOM + 0.02% CUSM group; 7.22 g in the

TABLE I – BODY, LIVER, RELATIVE LIVER, EPIDIDYMAL FAT AND PANCREAS WEIGHTS IN EACH GROUP OF MALE MICE (+/+, *db*/+ AND *db*/*db*) THAT RECEIVED AOM AND CUSM

Group no.	Treatment	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body wt.)	Epididymal fat weight (g)
1 (+/+)	AOM alone (9)	25.3 ± 1.1	1.23 ± 0.14	4.79 ± 0.42	0.39 ± 0.05
2 (+/+)	AOM + 0.02% CUSM (9)	23.8 ± 2.7	1.02 ± 0.20	4.25 ± 0.51	0.39 ± 0.15
3 (+/+)	AOM + 0.1% CUSM (9)	24.6 ± 1.3	1.15 ± 0.10	4.68 ± 0.29	0.37 ± 0.07
4 (+/+)	AOM + 0.5% CUSM (9)	24.7 ± 1.4	1.16 ± 0.06	4.70 ± 0.25	0.40 ± 0.11
5 (*db*/+)	AOM alone (10)	29.0 ± 1.5	1.29 ± 0.10	4.45 ± 0.26	0.65 ± 0.15
6 (*db*/+)	AOM + 0.02% CUSM (10)	29.6 ± 1.5	1.26 ± 0.18	4.24 ± 0.58	0.75 ± 0.12
7 (*db*/+)	AOM + 0.1% CUSM (10)	29.2 ± 1.2	1.25 ± 0.10	4.27 ± 0.28	0.74 ± 0.14
8 (*db*/+)	AOM + 0.5% CUSM (10)	28.9 ± 1.9	1.29 ± 0.11	4.47 ± 0.32	0.61 ± 0.14
9 (*db*/*db*)	AOM alone (10)	47.3 ± 5.0[1,2]	2.92 ± 0.53[3,4]	6.18 ± 0.73[3,4]	2.21 ± 0.45[1,2]
10 (*db*/*db*)	AOM + 0.02% CUSM (10)	46.6 ± 3.8[3,4]	2.79 ± 0.70[5,6]	5.96 ± 0.80[6,7]	2.28 ± 0.25[4,5]
11 (*db*/*db*)	AOM + 0.1% CUSM (10)	46.8 ± 7.3[5,6]	2.65 ± 0.47[1,2]	5.69 ± 0.92[1,2]	2.16 ± 0.50[6,7]
12 (*db*/*db*)	AOM + 0.5% CUSM (10)	45.2 ± 5.2[8,9]	2.47 ± 0.67[8,9]	5.43 ± 0.96[10]	2.28 ± 0.32[9,10]

All values are Mean ± SD.
Statistic analysis was done by Bonferroni Multiple Comparisons Test.
Values in parentheses in Column 2 indicate the number of mice examined.
[1]Significantly different from Group 4 ($p < 0.001$).–[2]Significantly different from Group 8 ($p < 0.001$).–[3]Significantly different from Group 2 ($p < 0.001$).–[4]Significantly different from Group 6 ($p < 0.001$).–[5]Significantly different from Group 3 ($p < 0.001$).–[6]Significantly different from Group 7 ($p < 0.001$).–[7]Significantly different from Group 3 ($p < 0.05$).–[8]Significantly different from Group 1 ($p < 0.001$).–[9]Significantly different from Group 5 ($p < 0.001$).–[10]Significantly different from Group 5 ($p < 0.05$).

AOM + 0.1% CUSM group and 7.25 g in the AOM + 0.5% CUSM group) were 1.25–1.34 times ($p < 0.01$ to $p < 0.001$) greater than other two genotypes (+/+ and *db*/+), regardless of treatments. The average body weights at the termination of the study were high in order of the *db*/*db* mice, the *db*/+ mice and the +/+ mice, as shown in Table I. Although the body weights of *db*/*db* mice were statistically higher ($p < 0.001$) than those of *db*/+ and +/+ mice, there was no significant difference among the treatment groups of each genotype. The liver and relative liver weights of *db*/*db* mice were greater than those of *db*/+ and +/+ mice, but the values did not significantly differ among the treatments groups of this genotype (Table I). The epididymal fat weight was heavy in the order of *db*/*db*, *db*/+ and +/+; the weight was insignificant among the treatment groups in each genotype (Table I). There were no significant differences regarding the mean pancreatic weight among the genotypes (data not shown). No clinical signs for the toxicity of CUSM were observed during the study.

Frequency of ACF and BCACs

At the end of the study, all the mice that received AOM developed colonic ACF and BCACs. Table II summarizes the data on colonic ACF formation. Regarding the mean number of ACF/colon in the AOM alone groups, the mean number of *db*/*db* mice was significantly higher ($p < 0.001$) than that of *db*/+ or +/+ mice. In comparison to the AOM alone group, the dietary administration with CUSM significantly reduced the number of ACF in all the genotypes: *db*/*db* mice, 53% reduction ($p < 0.001$) at a dose level of 0.02% CUSM, 54% reduction ($p < 0.001$) at a dose level of 0.1% CUSM and 59% reduction ($p < 0.01$) at a dose level of 0.5% CUSM; *db*/+ mice, 48% reduction ($p < 0.01$) at a dose level of 0.1% CUSM, 38% reduction ($p < 0.05$) at a dose level of 0.5% CUSM and +/+ mice, 45% reduction ($p < 0.05$) at a dose level of 0.1% CUSM and 62% reduction ($p < 0.001$) at a dose level of 0.5% CUSM. In addition, the percentages of ACF consisting of more than 4 aberrant crypts in all the CUSM-feeding groups in the *db*/*db* mice were significantly smaller (36% reduction by 0.02% CUSM, $p < 0.01$; 30% reduction by 0.1% CUSM, $p < 0.05$ and 47% reduction by 0.5% CUSM, $p < 0.001$) than that of AOM alone group (Table II). Although dietary administration with CUSM reduced the percentages of ACF consisting of more than 4 aberrant crypts in the *db*/+ and +/+ mice, the differences were insignificant.

BCACs also developed in the colon of all the genotypes of mice that received AOM alone, and the frequency per cm^2 of colonic mucosa was high in order of *db*/*db*, +/+ and *db*/+ mice (Table III). The dietary administration with CUSM at the highest dose (0.5%) significantly reduced the number of BCACs in the +/+ (65% reduction, $p < 0.05$) and *db*/*db* mice (74% reduction, $p < 0.001$). CUSM at a dose of 0.1% also significantly lowered the number of BCACs in *db*/*db* mice (53% reduction, $p < 0.001$).

Immunohistochemical analysis of Ob-R and IGF-1R

The immunohistochemical expression of Ob-R and IGF-1R was observed in the cytoplasm and nuclei of cryptal cells. Their expression was relatively strong in the nuclei of atypical cells in BCACs, when compared with their surrounding cryptal cells. Feeding with CUSM did not influence the stainability of Ob-R and IGF-1R (data not shown).

PCNA-labeling index

PCNA-labeling index was determined in BCACs that developed in the *db*/*db* mice (Groups 9 through 12). As illustrated in Figure 1, the mean PCNA-labeling indices of Group 11 (AOM + 0.1% CUSM, $p < 0.05$) and Group 12 (AOM + 0.5% CUSM, $p < 0.005$) were significantly lower than that of Group 9 (AOM alone). The values of Groups 9 and 10 (AOM + 0.02% CUSM) were comparable.

Histopathology and morphometric analysis in the liver

A histopathological examination of the liver revealed the occurrence of fatty metamorphosis [Fig. 2A-(*c*)] and fibrosis [Fig. 2B-(*c*)] in the *db*/*db* mice that received AOM alone, in contrast to the +/+ [Figs. 2A-(*a*) and 2B-(*a*)] and *db*/+ mice [Figs. 2A-(*b*) and 2B-(*b*)]. When the *db*/*db* mice were fed with 0.5% CUSM, these histopathological alterations (Fig. 3*a* and 3*b*) were inhibited ($p < 0.001$ for fatty metamorphosis and $p < 0.05$ for liver fibrosis).

Serum levels of cholesterol, triglycerides, glucose, insulin and leptin

The serum concentrations of total cholesterol, triglycerides, glucose, insulin and leptin are listed in Table IV. All the measurements in the *db*/*db* mice were higher than those of *db*/+ and +/+ mice. The dietary administration with CUSM did not significantly affect the serum levels of total cholesterol, glucose, insulin and leptin in all the genotypes. However, the serum level of triglycerides significantly decreased in the *db*/*db* mice ($p < 0.05$), when fed with the diet containing 0.5% CUSM (Table IV).

Discussion

The results of the current study confirmed the high susceptibility of AOM-induced colon carcinogenesis in the obese/diabetic *db*/*db* mice in our previous findings.[29] The high susceptibility in the *db*/*db* mice may be related to the increases in the body weight and the serum levels of total cholesterol, triglycerides, glucose, insulin and leptin, thus suggesting a positive association between