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Dietary Conjugated Linolenic Acid Inhibits Azoxymethane-induced Colonic Aberrant Crypt Foci in Rats

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The modifying effects of dietary feeding of conjugated linolenic acid (CLN) isolated from the seeds of bitter gourd (*Momordica charantia*) on the development of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) were investigated in male F344 rats to predict its possible cancer chemopreventive efficacy. The effect of CLN on the proliferating cell nuclear antigen (PCNA) index in colonic ACF was also examined. Rats were given subcutaneous injections of AOM (20 mg/kg body weight) once a week for 2 weeks to induce ACF. They also received the experimental diet containing 0.01%, 0.1% or 1% CLN for 5 weeks, starting one week before the first dosing of AOM. AOM exposure produced a substantial number of ACF (108±21/rat) at the end of the study (week 4). Dietary administration of CLN caused a significant reduction in the frequency of ACF: 87±14 (19.4% reduction, $P<0.05$) at a dose of 0.01%, 69±28 (36.1% reduction, $P<0.01$) at a dose of 0.1% and 40±6 (63.0% reduction, $P<0.001$) at a dose of 1%. Also, CLN administration lowered the PCNA index and induced apoptosis in ACF. These findings might suggest possible chemopreventive activity of CLN in the early phase of colon tumorigenesis through modulation of cryptal cell proliferation activity and/or apoptosis.

Key words: Conjugated linolenic acid — Chemoprevention — Aberrant crypt foci — PCNA index — Apoptosis

One-third of human cancers might be associated with dietary habits and lifestyle,[1)] and dietary factors are known to be among the most important modulators of colon cancer development. The amount and type of dietary fat consumed are of particular importance.[2–5)] Epidemiological studies indicate that consumption of fish and fish oil correlates with a reduced risk of colon cancer.[6)] Fish oil is rich in the *n*–3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) (20:5) and docosahexaenoic acid (DHA) (22:6). Studies in humans and experimental animals indicate a protective effect of *n*–3 PUFA (fish, fish oils or EPA ethyl ester), and the mechanism of protection is thought to be mainly related to interference with biosynthesis of two-series prostaglandins (PGs) from arachidonic acid (AA) (22:4, *n*–6).[7)] Our recent work[8)] also demonstrated that tuna oil rich in DHA and vitamin D_3 inhibits azoxymethane (AOM)-induced rat aberrant crypt foci (ACF), which are putative precursor lesions for colonic adenocarcinoma.[9)] Limited studies of α-linolenic acid (α-LN) (18:3, *n*–3), the parent fatty acid of the *n*–3 family, provided some promising results. Dietary feeding of perilla and flaxseed oils, both rich sources of α-LN decreased chemically induced colonic neoplasms and ACF in rat colon carcinogenesis models.[10–12)] These results are consistent with competitive exclusion of *n*–6 PUFA from membrane phospholipids and associated reductions in PGE_2 concentration in colonic mucosa.[12)]

Other fatty acids, including certain *n*–6 PUFAs and their derivatives, may also have antitumorigenic properties. Conjugated linoleic acid (CLA, conjugated diene) refers collectively to several positional and geometric isomers of linoleic acid (LA) (18:2, *n*–6) in which the double bonds are in conjugation, typically at positions 9 and 11 or 10 and 12. CLA, predominantly as dienoic fatty acid 9(*Z*),11(*E*)-18:2(*n*–7), occurs naturally in small amounts in cooked meats, dairy products and ruminant meats.[13)] CLA is potently antitumorigenic in chemically induced rat mammary tumorigenesis,[14)] and possesses anti-tumor promoting action in two-stage mouse skin tumorigenesis.[15)] CLA exerts its chemopreventive effect by inhibiting metabolism of LA to AA, thereby decreasing biosynthesis of AA-derived PGs.[16, 17)] Dietary CLA could exert its chemopreventive effects at relatively high doses with a range of 0.1–1%.[18)] When compared with studies examining the efficacy of CLA on mammary carcinogenesis, evidence for chemoprevention against colon cancer is less definitive. For example, in rats gavaged with CLA, fewer ACF were induced by heterocyclic amines.[19, 20)]

Other types of conjugated PUFAs with biological activity[21–23)] occur in certain natural products.[24, 25)] They include conjugated trienoic fatty acids, such as conjugated octadecatetraenoic acid (*cis*(*c*)9,*trans*(*t*)11,*t*13,*c*15-18:4;α-

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parinaric acid) in garden balsam seed oil and *c*9,*t*11,*t*13-18:3 in bitter gourd seed oil. The latter acts as an antioxidant.[26] Although conjugated PUFAs including CLA are usually present in amounts of less than 1% in natural products, some seed oils contain extremely high amounts (40–50%) of conjugated linolenic acid (CLN, conjugated triene).[27] Tung, bitter gourd, pomegranate, catalpa, and pot marigold seed oils contain 42–83% of α-eleostearic (*c*9,*t*11,*t*13-CLN), punicic (*c*9,*t*11,*c*13-CLN), catalpic (*t*9,*t*11,*c*13-CLN), and calendic (*t*8,*t*10,*c*12-CLN) acids.[28] Among these seeds, bitter gourd and pomegranate are edible plants, and the former is an important food material in South-East Asia. Recently, a cytotoxic effect of CLN on various human cancer cell lines has been reported.[22] It was found that alkaline isomerized α-LN and tung oil fatty acids were cytotoxic to cancer cell lines, suggesting that conjugated trienoic fatty acids are more cytotoxic to cancer cell lines than the conjugated dienoic fatty acid, CLA. However, isomerized LA is a mixture of unreacted LN and CLN having conjugated dienoic and trienoic double bonds. Tung oil fatty acids are also a mixture of α-eleostearic acid and nonconjugated fatty acid. Therefore, the cytotoxic effect of each positional and geometrical CLN isomer should be elucidated.

Since the cytotoxicity of *c*9,*t*11,*t*13-CLN, *c*9,*t*11,*c*13-CLN, and *t*9,*t*11,*c*13-CLN is much greater than that of *t*8,*t*10,*c*12-CLN in our recent *in vitro* study on human leukemic cells,[28] in the present study, we examined the modulatory effects of *c*9,*t*11,*t*13-CLN isolated bitter gourd (*Momordica charantia*) oil on the development of ACF in rats treated with a colon carcinogen, AOM. In this short-term *in vivo* experiment, the proliferating cell nuclear antigen (PCNA) index and the apoptotic index in ACF were evaluated to assess whether CLN modifies cell proliferation activity and induces apoptosis in ACF, since certain chemopreventive agents exerts their inhibitory action via reduction of the PCNA index,[29] and induction of apoptosis.[30]

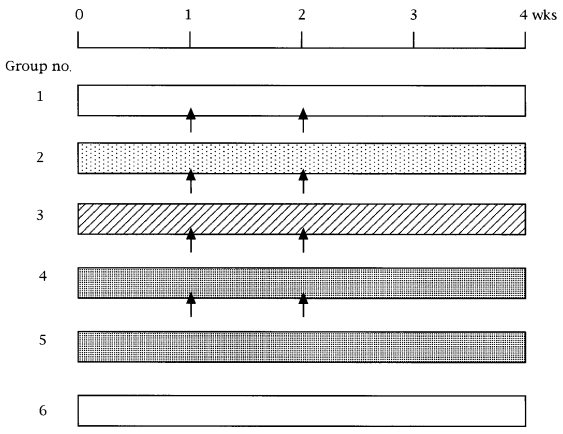

Fig. 1. Experimental protocol. ↑: azoxymethane (20 mg/kg b.w., s.c.). □: basal diet, CE-2. ▭: 0.01% CLN in diet. ▨: 0.1% CLN in diet. ▦: 1% CLN in diet.

MATERIALS AND METHODS

Oil source for CLN with conjugated trienes Seeds of bitter gourd (*M. charantia*) were obtained from Tohoku Seed Co., Tochigi. The seeds was ground to a powder with an electric mill. Total lipids were extracted by the method described by Folch *et al*.[31] and Suzuki *et al*.[28] The fatty acid composition of total lipids was analyzed by capillary gas chromatography after conversion of fatty acyl groups in each lipid to their methyl esters with sodium methoxide. Component peaks were identified by reference to known standards[27] and were quantified by a Shimadzu Chromatopac C-R6A integrator (Shimadzu Seisakusho Co., Ltd., Kyoto). Bitter gourd seeds contained 41.0% total lipids per wet weight. Thin layer chromatographic analysis showed that the total lipids were mainly composed of triacylglycerols. The fatty acid profile of the total lipids was generally in harmony with that described in the previous paper,[27] with a very high level (60.2%) of *c*9,*t*11,*t*13-18:3 and small amounts of other CLN isomers, namely, *c*9,*t*11,*c*13-18:3 (0.6%) and *t*9,*t*11,*t*13-18:3 (0.3%). These lipids also contained a large amount (27.2%) of 18:0 and modest amounts of 18:1*n*−9 (5.9%) and 18:2*n*−6 (3.8%). Thus, the most important characteristic of bitter gourd seed lipids is the high amounts of conjugated trienoic fatty acids such as *c*9,*t*11,*t*13-18:3.

Animals, chemicals, and diets Male F344 rats (Charles River Japan, Inc., Kanagawa) aged 4 weeks were used for ACF assay. The animals were maintained in the 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 drinking water and a basal diet, AIN-76A,[32] 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 (Fig. 1). Beginning at 5 weeks of age, all animals were fed each of the four different experimental diets. The rats in groups 1 and 6 were fed the diet containing 5% corn oil. Group 2 were fed the diet containing 0.01% CLN and 4.99% corn oil. Group 3 were fed the diet containing 0.1% CLN and 4.9% corn oil. Groups 4 and 5 were fed to the diet containing 1% CLN and 4% corn oil. All rats were provided with the experimental diets and tap water *ad libitum*, and weighed weekly. Four experimental diets based on the AIN-76 formulation[32] containing various levels of CLN (0%, 0.01%, 0.1% or 1% by weight of diet) were made on a weekly basis and stored at −20°C under a nitrogen atmo-

sphere in airtight containers for no longer than a week. The composition of the diets is shown in Table I. The fatty acid composition (weight %) of CLN, of more than 99% purity, from bitter gourd seed oil used for this study was 1.3% 16:0, 27.4% 18:0, 5.9% 18:1*n*−9, 3.8% 18:2(*c*9,*c*12), 0.6% 18:3(*c*9,*t*11,*c*13), 60.3% 18:3(*c*9,*t*11,*t*13), and 0.3% 18:3(*t*9,*t*11,*t*13).[28] At 6 weeks of age, all animals were given subcutaneous injection of AOM (20 mg/kg body weight) once a week for 2 weeks. AOM for ACF induction was purchased from Sigma Chemical Co. (St. Louis, MO). All animals were killed 2 weeks after the last administration of AOM, and complete necropsies were performed. Colons of 5 rats each from groups 1–4 and those of 2 rats each from groups 5 and 6 were used to determine the presence of ACF. The distal colons (4 cm from the anus) of these animals were used to measure PCNA labeling and apoptosis of the ACF and surrounding crypts. The livers of all rats were used to determine the effects of dietary CLN on the lipid composition of liver.

Determination of ACF The frequency of ACF was determined according to the method described in our previous report.[33] 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. Fixed colons were dipped in a 0.5% solution of methylene blue in distilled water for 30 s, briefly washed with the distilled water, and placed on a microscope slide with the mucosal surface up. Using a light microscope at a magnification of ×40, ACF were distinguished from the surrounding normal-appearing crypts by their increased size.[9]

Immunohistochemistry for PCNA Immunohistochemical staining for PCNA was performed by the avidin-biotin complex method (Vecstain Elite ABC Kit, Vector, Burlingame, CA). Tissue sections were deparaffinized with xylene, hydrated through a graded ethanol series, immersed in 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature to block endogenous peroxidase activity, and then washed in phosphate-buffered saline (pH 7.2). Following incubation with normal rabbit serum at room temperature for 10 min to block background staining, the sections were incubated with an anti-PCNA antibody (mouse monoclonal PC10, Dako Co., Kyoto; 1:100 dilution) for 12 h in a humidified chamber at room temperature. They were then reacted with 3,3′-diaminobenzidine and counterstained with Harris' hematoxylin. For determination of PCNA-positive index, 10 full-length crypts (aberrant crypts, normal-appearing crypts or normal crypts) of each colon were examined.

Table I. Composition of Experimental Diets[a]

Diet ingredients	Control (0% CLN)	0.01% CLN	0.1% CLN	1% CLN
Casein	20.0[b]	20.0	20.0	20.0
DL-Methionine	0.3	0.3	0.3	0.3
Corn starch	15.0	15.0	15.0	15.0
Dextrose	50.0	50.0	50.0	50.0
Cellulose	5.0	5.0	5.0	5.0
Corn oil	5.0	4.99	4.9	4.0
CLN	0	0.01	0.1	1.0
Mineral mix[c]	3.5	3.5	3.5	3.5
Vitamin mix[c]	1.0	1.0	1.0	1.0
Choline bitartrate	0.2	0.2	0.2	0.2

CLN, conjugated linolenic acid.
a) Diets were prepared on the premises and stored in sealed containers under inert conditions for no longer than a week at −20°C.
b) Values are g/100 g diet.
c) AIN-76A.

Table II. Effect of Conjugated Linolenic Acid (CLN) on Body Weight Gain and Food Intake of Rats

Group no.	Treatment	Body weight gain (%)	Mean food intake (g/rat/day)	Food efficacy[a]
1	AOM alone (8)	54.7±1.4[b]	12.8±2.0	8.13±0.60
2	AOM+0.01% CLN (8)	53.1±2.8	12.4±2.1	7.69±1.02
3	AOM+0.1% CLN (8)	52.3±0.8[c]	12.6±2.2	7.23±0.22[d]
4	AOM+1% CLN (8)	53.6±1.6	12.4±1.9	7.63±0.44
5	1% CLN (4)	56.3±1.2[e]	12.5±1.4	8.45±0.20[e]
6	None (4)	54.0±0.9	12.9±1.9	7.90±0.31

a) Body weight gain (g)/food intake (g/day). AOM, azoxymethane; CLN, conjugated linolenic acid.
b) Mean±SD.
c, *d*) Significantly different from group 1 by Student's *t* test or Welch's *t* test (*c*) $P<0.001$ and *d*) $P<0.005$).
e) Significantly different from group 6 by Student's *t* test ($P<0.05$).

Table III. 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)	190±12[a)]	11.1±1.4	5.82±0.67
2	AOM+0.01% CLN (8)	179±15	9.4±0.8[b)]	5.26±0.32
3	AOM+0.1% CLN (8)	174±5[b)]	9.5±1.5[c)]	5.44±0.93
4	AOM+1% CLN (8)	176±7[d)]	10.3±1.0	5.85±0.52
5	1% CLN (4)	188±6	9.7±0.7	5.16±0.36
6	None (4)	189±8	10.5±0.7	5.58±0.25

AOM, azoxymethane; CLN, conjugated linolenic acid.
a) Mean±SD.
b–*d*) Significantly different from group 1 by Student's *t* test or Welch's *t* test (*b*) $P<0.01$, *c*) $P<0.05$, and *d*) $P<0.02$).

Table IV. Incidence of ACF in Rats Treated with AOM and/or Conjugated Linolenic Acid (CLN)

Group no.	Treatment (No. of rats examined)	Total no. of ACF/colon	Total no. of ACs/colon	No. of ACs/focus	% of ACF containing 4 or more ACs
1	AOM alone (8)	108±21[a)]	215±42	1.95±0.05	8.57±3.39
2	AOM+0.01% CLN (8)	87±14[b)]	164±30[c)]	1.89±0.05[b)]	6.18±1.36
3	AOM+0.1% CLN (8)	69±28[d)]	130±52[e)]	1.88±0.05[c)]	8.32±1.46
4	AOM+1% CLN (8)	40±6[f)]	69±10[f)]	1.76±0.08[f)]	3.89±2.63[d)]
5	1% CLN (4)	0	0	0	0
6	None (4)	0	0	0	0

ACF, aberrant crypt foci; ACs, aberrant crypts; AOM, azoxymethane.
a) Mean±SD.
b–*f*) Significantly different from group 1 by Student's *t* test or Welch's *t* test (*b*) $P<0.05$, *c*) $P<0.02$, *d*) $P<0.01$, *e*) $P<0.005$, and *f*) $P<0.001$).

The number and the position of PCNA positively stained nuclei in each crypt column were recorded and expressed as PCNA-positive index (number of positive stained nuclei ×100/total number of nuclei counted). The observer (T. T.) was unaware of the group to which the specimens belonged.

Measurement of apoptotic index The frequency of apoptotic cells in ACF and normal-appearing crypts was determined using the morphological criteria reported.[34] Colonic mucosa was stained with Feulgen/fast green to score the apoptotic index. Ten randomly chosen ACF or normal-appearing crypts per rat were observed under a light microscope (×400). Apoptotic cells were identified by the presence of condensed chromatin and spherical apoptotic bodies containing nuclear materials.[34] Apoptotic index (%) was calculated as the mean number of apoptotic cells/number of cells in the crypt section ×100.

Lipid extraction and analysis At necropsy, the liver was excised and weighed, then the caudate lobe was removed and fixed in 10% buffered formalin for histological examination. Remaining lobes of the liver were frozen at −80°C until lipid analyses were performed. Liver lipids were extracted with chloroform/methanol (2:1, v/v) as described previously by Folch *et al.*[31] Component peaks were identified by comparison with standard fatty acid methyl ester[35] and quantified by a Shimadzu Chromatopac C-R6A integrator (Shimadzu Seisakusho Co., Ltd.). The identification of CLA isomers was confirmed by using GC-mass spectrometry after conversion of the methyl esters to dimethyloxazoline derivatives.[36] The analysis of fatty acid composition was done more than two times for each sample and there was no significant difference between results for the same sample. Data are presented as means±SD.

Statistical evaluation Where applicable, data were analyzed using one-way ANOVA, Student's *t* test or Welch's *t* test with $P<0.05$ as the criterion of significance.

RESULTS

General observation All animals remained healthy throughout the experimental period. Body weight gain (%) and mean food consumption of rats (g/rat/day) in various groups are shown in Table II. Mean body weight gains of rats in groups 3 (AOM + 0.1% CLN) and 5 (1% CLN) were significantly smaller than those of groups 1 (AOM) and 6 (untreated), respectively ($P<0.001$ and $P<0.05$). Food intake did not differ significantly among the groups. Calculated food efficacies of all groups except group 3 were comparable. Mean body, liver, and relative liver weights (g/100 g body weight) of all groups are summarized in Table III. The mean body weights of groups 3 and 4 were significantly lower than that of group 1 ($P<0.01$ and $P<0.02$, respectively). The mean liver weights of groups 2 and 3 were significantly smaller than that of group 1 ($P<0.01$ and $P<0.05$, respectively). However, mean relative liver weights did not differ significantly among the groups. Histology of the liver revealed no morphological alterations, such as fatty liver.

Frequency of ACF Table IV summarizes the data on colonic ACF (Fig. 2, a and b) analysis. All rats belonging to groups 1 through 4, initiated with AOM, developed ACF. The mean number of ACF/colon in group 1 was 108±21. The dietary administration of CLN at various doses caused significant inhibition of ACF formation: 87±14 (19% reduction, $P<0.05$) at a dose of 0.01% (group 2), 69±28 (36% reduction, $P<0.01$) at a dose of 0.1% (group 3), and 40±6 (63% reduction, $P<0.001$) at a dose of 1% CLN (group 4). Furthermore, there were significant decreases in the total number of ACs per colon ($P<0.02$, $P<0.005$ and $P<0.001$) and the number of ACs per focus ($P<0.05$, $P<0.02$ and $P<0.001$) in these groups. Also, the percentages of ACF that consisted of more than 4 crypts in group 4 was significantly smaller than that of

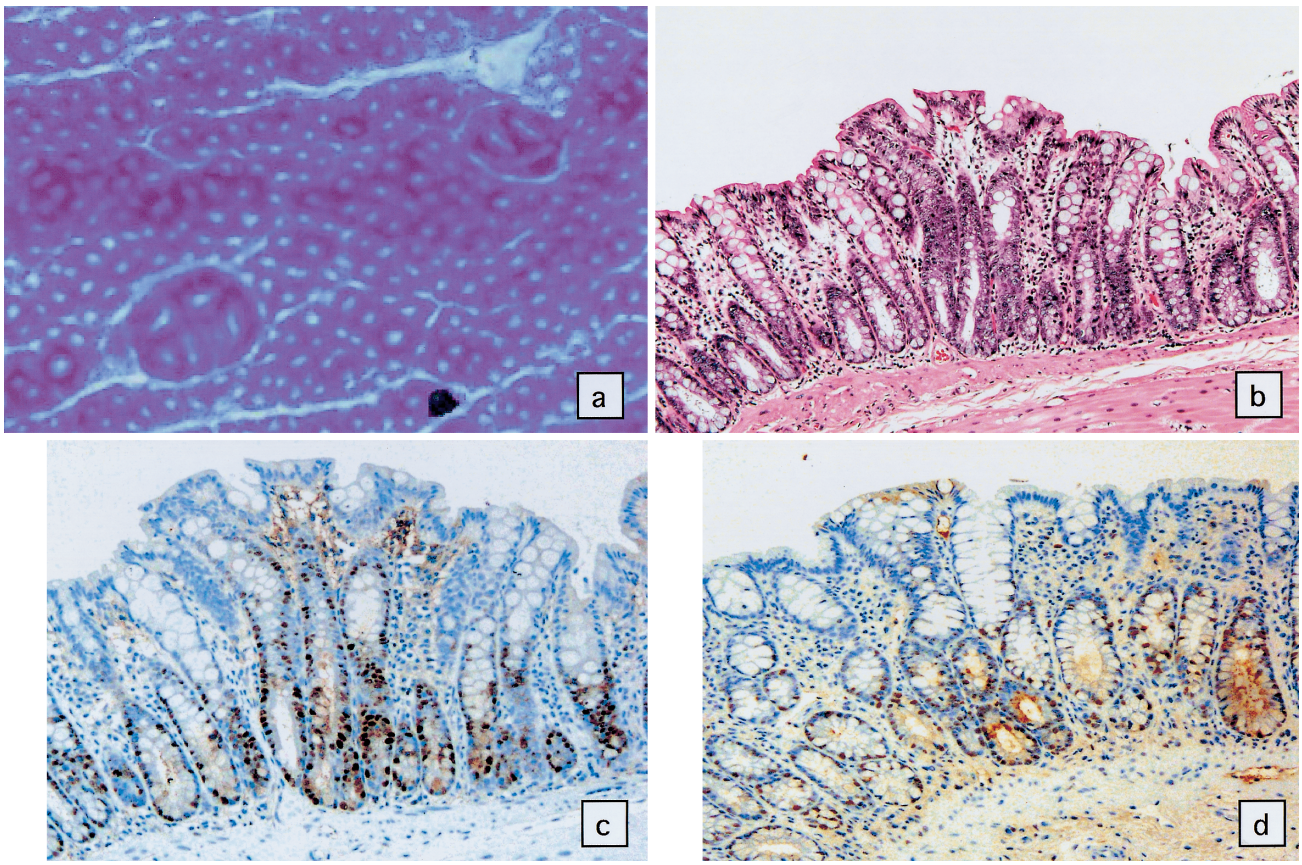

Fig. 2. Morphology and PCNA immunohistochemistry of ACF. (a) ACF on methylene-blue-stained colonic mucosa of a rat in group 1; (b) ACF on hematoxylin and eosin-stained section from a rat in group 1; (c) PCNA immunohistochemistry of ACF of a rat from group 1; and (d) PCNA immunohistochemistry of ACF of a rat from group 4. Original magnification, (a) ×4, (b) ×10, (c) ×10, and (d) ×10.

group 1 ($P<0.01$). In groups 5 and 6, which were given a CLN diet alone and untreated, respectively, there were no microscopically observable changes, including ACF, in colonic morphology.

PCNA labeling index in ACF and normal-appearing colonic crypts The PCNA labeling indices in ACF (Fig. 2, c and d) and normal-appearing crypts are presented in Table V. PCNA-labeling index was higher in the ACF than in the surrounding normal-appearing crypts in all rats in groups 1–4. Dietary feeding of CLN at various doses reduced the mean PCNA-labeling indices in ACF compared with group 1, and the reduction in group 4 was statistically significant ($P<0.05$). The PCNA-labeling index of normal-appearing crypts of this group was also significantly lower than in group 1 ($P<0.05$). Feeding of 1% CLN did not affect the PCNA-labeling index in normal-appearing crypts of rats in group 5, when compared with that of untreated rats (group 6).

Table V. PCNA-labeling and Apoptotic Indices in ACF and Colonic Crypts of Rats Treated with AOM and/or Conjugated Linolenic Acid (CLN)

Group no.	Treatment	PCNA-labeling index (%)		Apoptotic index (%)	
		ACF	Surrounding normal-appearing crypts	ACF	Surrounding normal-appearing crypts
1	AOM alone	22.4±5.5[a)] (10)	16.5±7.1[b)] (10)	1.70±0.55 (10)	0.47±0.12 (10)
2	AOM+0.01% CLN	18.2±5.7 (10)	13.9±2.5 (10)	2.03±0.33 (10)	0.46±0.11 (10)
3	AOM+0.1% CLN	17.2±7.9 (10)	14.0±2.1 (10)	2.20±0.43[c)] (10)	0.49±0.12 (10)
4	AOM+1% CLN	16.0±7.6[c)] (10)	11.2±2.0[c)] (10)	2.15±0.23[c)] (10)	0.46±0.15 (10)
5	1% CLN	—	10.7±2.4 (10)	—	0.38±0.19 (10)
6	None	—	9.9±3.5 (10)	—	0.37±0.13 (10)

PCNA, proliferating cell nuclear antigen; AOM, azoxymethane; ACF, aberrant crypt foci.
No. in parentheses of the data are no. of ACF or crypts examined.
a) Mean±SD.
b) Significantly different from group 6 by Welch's *t* test ($P<0.05$).
c) Significantly different from group 1 by Student's *t* test or Welch's *t* test ($P<0.05$).

Table VI. Effects of Diets Containing Conjugated Linolenic Acid (CLN) on Fatty Acid Composition of Liver Lipids

Group no.	Treatment	Fatty acids (wt%)									
		16:0	16:1*n*−7	18:0	18:1*n*−7	18:1*n*−9	18:2*n*−6	18:2 (*c*9, *t*11)	20:4*n*−6	22:5*n*−3	22:6*n*−3
1	AOM alone	23.5±0.8[a)]	4.3±0.7	14.8±1.4	5.8±0.5	14.4±2.6	11.2±0.8	ND	18.2±2.2	2.1±0.3	1.8±0.2
2	AOM+ 0.01% CLN	25.2±0.8	4.8±0.7	15.4±0.9	6.1±0.2	13.8±2.1	8.2±0.8[b)]	0.2±0.0	18.5±1.3	2.0±0.1	1.9±0.2
3	AOM+ 0.1% CLN	25.2±0.8	4.7±0.8	14.9±1.0	5.9±0.6	14.9±1.8	8.0±0.8[b)]	0.1±0.0	17.7±1.7	1.8±0.1	2.0±0.2
4	AOM+ 1% CLN	24.8±1.0	4.5±0.3	16.0±0.4	5.7±0.5	14.3±1.2	8.1±0.7[b)]	0.8±0.2[c)]	17.7±0.9	1.9±0.2	1.6±0.1
5	1% CLN	23.5±0.9	4.1±0.4	15.6±1.3	5.8±0.4	12.8±0.7	7.9±1.0	0.5±0.1	18.9±1.7	1.8±0.1	1.9±0.2
6	None	24.4±2.0	4.3±1.4	15.3±1.7	6.2±0.4	13.0±2.1	10.1±1.5	ND	18.8±2.2	1.7±0.2	1.7±0.1

a) Mean±SD.
b) Significantly different from group 1 by Student's *t* test ($P<0.001$).
c) Significantly different from groups 2 and 3 by Student's *t* test ($P<0.01$).
ND means not detected in measurable quantities.

Apoptotic index Data on apoptotic index are given in Table V. Apoptotic index was generally greater in ACF than in the surrounding normal-appearing crypts, as was the case for PCNA-labeling index. The apoptotic cells were mostly in the lower-third of the crypts in all rats examined. Feeding of CLN diets increased the apoptotic index in ACF and the increases in groups 3 (AOM + 0.1% CLN) and 4 (AOM + 1% CLN) were statistically significant ($P<0.05$) when compared with group 1 (AOM). On the other hand, CLN treatment did not modify the apoptotic index in the surrounding normal-appearing crypts.

Lipid analysis The fatty acid profiles of the lipids from liver are shown in Table VI. Although CLN diets contained over 60% of CLN isomer (*c*9,*t*11,*t*13-18:3), no CLN isomer was detected in the liver of rats fed CLN diets at various doses. On the other hand, the content of CLA (*c*9,*t*11-18:2) in the liver lipids of rats treated with AOM and 1% CLN (group 4), was significantly greater than in groups 2 (AOM + 0.01% CLN) and 3 (AOM + 0.1% CLN) ($P<0.01$).

DISCUSSION

The results described here clearly indicate that dietary administration of CLN at various doses (0.01%, 0.1% or 1% in diet) significantly inhibits AOM-induced ACF formation in male F344 rats. Moreover, percentages of ACF that consisted of 4 or more aberrant crypts were significantly reduced by feeding of the diet containing 1% CLN. These findings, that dietary CLN at all doses effectively suppresses the early phase of chemically-induced rat colon tumorigenesis and 1% CLN in the diet significantly inhibits AOM-induced large ACF, may suggest inhibition 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.[9, 37, 38] A recent study by Rao *et al.*[5] also suggested that a high-fat mixed-lipid diet increases both the total number and the multicrypt foci of ACF, and this results in its exceptional promoting effect on AOM-induced rat colon carcinogenesis.

Dietary restriction can modulate both spontaneous and chemically induced carcinogenesis.[39] In the current study, mean body weight of rats given AOM and 0.1% (group 3) or 1% CLN diet (group 4) was significantly lower than that of the AOM-alone group (group 1). This may reflect the development of ACF. However, the relative liver weights (g/100 g body weight) were comparable to that of animals treated with AOM alone. Moreover, mean body, liver, and relative liver weights of rats given 1% CLN diet alone (group 5) were similar to those of an untreated control (group 6). Therefore, suppressing effects of CLN observed in this study may not be due to alteration of body weight gain.

There have been a few studies on the modifying effects of conjugated fatty acids on colon carcinogenesis. CLA was reported to inhibit mainly mammary and skin carcinogenesis.[14, 15] The mechanism through which CLA inhibits carcinogenesis is suspected to be a decrease in arachidonic acid in the liver of SENCAR mice fed a CLA diet[40] and a decrease in LA metabolites in mammary glands of rats.[16] Studies on the modifying effects of CLA on colonic ACF induced by heterocyclic amines suggested that CLA suppresses heterocyclic amine-induced ACF and adduct formation by inhibiting carcinogen activation, via effects on both hepatic P450 enzymes (P450 1A1 and 1A2) and extrahepatic prostaglandin *H* synthase.[41] In the current study, we did not assay these enzymes. An ongoing long-term study, in which rats are being fed CLN diet either during or after AOM treatment, should elucidate the mechanisms through which CLN suppresses colon carcinogenesis.

In the current study, the PCNA-labeling indices in ACF and normal-appearing crypts were decreased by dietary feeding of CLN at a dose of 1%. Similar findings that certain retinoids and natural compounds have the ability to reduce the PCNA-labeling index in ACF and in normal-appearing crypts in addition to the ability to prevent ACF were reported.[29, 42–44] Cell proliferation plays an important role in multistage carcinogenesis with multiple genetic changes.[45] Eicosanoids, metabolites of AA through the lipoxygenase and cyclooxygenase pathways, possess a variety of biological activities. Some metabolites of both pathways cause hyper-proliferative responses.[46] Interestingly, feeding of CLN enhanced apoptotic cells in ACF in the present study, without affected the surrounding normal-appearing crypts. Thus, the inhibitory effect of CLN may be due to, in part, modification of cell proliferation and apoptosis induction.

In the fatty acid profiles of the lipids from liver, we did not detect any CLN isomer in the liver lipids from rat fed the CLN diets, which contained over 60% of CLN isomer (*c*9,*t*11,*t*13-18:3). On the other hand, CLA was found in these lipids and the content of the CLA isomer (*c*9,*t*11-18:2) was significantly greater in rats fed the 1.0% CLN diet than that in rats fed the 0.01% and 0.1% CLN diets, although the amounts were small. This may indicate that part of *c*9,*t*11,*t*13-18:3 in the CLN would be enzymatically converted to *c*9,*t*11-18:2. CLA is a possible chemopreventive agent against ACF formation.[19, 20] Though CLA used in previous studies[14, 47–49] contained a mixture of positional and geometrical isomers, the *c*9,*t*11-18:2 isomer is considered to be the active constituent. Therefore, the suppressing effect of CLN on ACF formation in the current study may in part be attributed to the *c*9,*t*11-18:2 isomer derived from *c*9,*t*11,*t*13-18:3 in the CLN diets. Furthermore, in this study, the contents of linoleic acid (18:2*n*−6) in the liver lipids of rats fed the CLN diets were signifi-

cantly lower than that of rats fed diet without CLN. This reduction in LA may also be correlated to the inhibitory effect of CLN on ACF formation. However, judging from the powerful inhibitory activity of CLN found in the present study, other factors, such as direct action of *c*9,*t*11,*t*13-18:3 on the early stage of colon carcinogenesis, should be considered.

The antioxidant activity of *c*9,*t*11,*t*13-18:3 is another possible explanation for inhibition of ACF formation by feeding the CLN diet, since another conjugated fatty acid with chemopreventive ability, CLA, may act through antioxidant mechanisms,[14, 48] prooxidant mechanisms,[50] inhibition of nucleotide synthesis,[51] and reduction of proliferative activity.[49] In compounds with more than two conjugated double bonds, conjugation increased the rate of oxidation. Thus, in the *in vivo* study, conjugated trienoic fatty acids are also likely to be more rapidly oxidized than linoleates by picking up more free radicals, thereby eliminating or reducing the formation of hydroperoxides. CLA at higher concentrations acts as a prooxidant rather than an antioxidant.[52] At 0.5%, *c*9,*t*11,*t*13-18:3 showed an antioxidant property that decreased with increase in its level. At higher concentrations it could possibly act as a prooxidant, like CLA.[52] Thus, it is possible that in this study CLN reduces the formation of hydroperoxides by lowering the generation of free radicals and peroxidation of PUFA occurring in cell membrane and other lipids.

In this study, we evaluated immunohistochemical expression of peroxisome proliferator-activated receptor (PPAR)-γ (data not shown). The expression in ACF was more robust than in normal-appearing colonic crypts. Recently, CLA was reported to act as a high-affinity ligand and activator of PPARs-α and -γ[53–55] Moya-Camarena *et al.*[56] demonstrated a hierarchy of binding affinity for certain CLA isomers in the order of *c*9,*t*11 > *t*10,*t*12 > *t*9,*t*11. In agreement with its high binding affinity, *c*9,*t*11-CLA was also the most efficacious PPAR-α activator, as determined in a PPAR-α receptor gene assay system. In addition, McCarty[54] suggested that part of CLA's anticarcinogenic activity is mediated by PPAR-γ activation in susceptible tumors. In the current study, dietary feeding of CLN slightly enhanced PPAR-γ expression in ACF, although we did not quantify the expression. These results are of interest, since we recently demonstrated that synthetic ligands for PPARs-α and -γ effectively inhibit AOM-induced ACF in rats[57] and a PPAR-γ ligand induced apoptosis in normal-appearing crypts.[54] Thus, it may be possible that CLN suppresses ACF formation via altering PPARs expression in ACF and/or colonic crypts.

In conclusion, the results of this study suggest that dietary CLN has a beneficial effect on chemically induced colonic preneoplastic progression in rats, and it may represent an effective dietary chemopreventive approach to disease management. A long-term bioassay is under way in our laboratory to confirm the results described here and to elucidate the exact mechanism(s) by which CLN inhibits the early phase of colon tumorigenesis. Our findings suggest that CLN from the seeds of bitter gourd (*M. charantia*) should enter preclinical studies for the prevention of colon cancer.

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