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Dietary conjugated linoleic acid increases the mRNA ratio of Bax/Bcl-2 in the colonic mucosa of rats

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Abstract

Previously we have shown that dietary conjugated linoleic acid (CLA) significantly decreased colon tumor incidence in rats injected with 1,2-dimenthylhydrazine (DMH). The present study was performed to explore the mechanisms responsible for the anticarcinogenic effect of CLA. Four groups of rats received either vehicle or intramuscular injections of DMH at the dose of 15 mg/kg body weight twice per week for 6 weeks and were fed a diet containing either 0% or 1.0% CLA *ad libitum* for 14 weeks. Dietary CLA decreased cellular proliferation and induced apoptosis in the colonic mucosa of both vehicle and DMH-treated rats. Mucosal levels of prostaglandin (PG) E_2, thromboxane B_2, and 1,2-diacylglycerol decreased in rats fed the 1% CLA diet, whereas cyclooxygenase-2 levels were not affected. Arachidonate content of mucosal phospholipids decreased significantly in rats fed the 1% CLA diet. Reverse transcriptase–polymer chain reaction analysis revealed that the Bax/Bcl-2 transcript ratio was significantly increased in rats fed 1% CLA. To examine whether the 1% CLA diet reduces tumor incidence, the DMH-treated rats were continuously fed the assigned diets for 30 weeks. Tumor incidence was significantly decreased in the CLA-fed group. In conclusion, our findings are consistent with the hypothesis that CLA decreases the incidence of colon cancer by decreasing cellular proliferation and inducing apoptosis of the colonic mucosa. These effects may be due in part to decreased PGE_2 levels and increased Bax/Bcl-2 ratios.

Keywords: CLA; Colon cancer; Cyclooxygenase; Cell proliferation; Bcl-2; Bax

1. Introduction

Colorectal cancer is one of the most common malignancies in the western world. Although surgical excision is the best option for treatment, many patients who undergo therapeutic resection will develop tumor recurrences. Therefore, there is increasing urgency to develop strategies to prevent this disease. Recent development in the understanding of diet in colon cancer etiology has raised expectations that this increasing knowledge might lead to improved cancer prevention. In this regard, the identification of dietary factors that can prevent colon cancer would show particular promise.

Conjugated linoleic acid (CLA) is a group of polyunsaturated fatty acids found in diary products, beef, and lamb. CLA exists as geometric and positional isomers of octadecadienoic acid with a variety of biological effects in experimental animal studies. There is strong evidence that dietary CLA is an effective anticancer agent with a number of cancer sites protected by CLA in animal models [1]. Therefore, it is necessary to study the signaling pathway of CLA and molecular targets that are responsible for the anticarcinogenic effect of CLA.

Human colonic mucosa as well as colon cancer shows a profile of prostanoid biosynthesis [2]. Cyclooxygenase (COX) catalyzes the oxygenation of arachidonic acid to prostaglandins, prostacyclins, and thromboxanes in mammalian cells. Two forms of the COX enzyme are known. COX-1 is the major enzyme form constitutively expressed and plays a role in thrombogenesis and the homeostasis of the gastrointestinal tract. COX-2 is a distinct isoform of COX-1 that is inducible and upregulated in pathological states including inflammation and neoplasia, and that has been associated with the elevated production of prostaglandin (PG) [3].

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It has been demonstrated that COX-2 expression is increased in human colorectal adenocarcinomas compared to normal adjacent colonic mucosa [2]. In addition, the relative levels of prostanoids were elevated in adenomas compared with normal appearing mucosa from the same patients. Furthermore, recent studies have reported a 40-50% reduction in the relative risk of colorectal cancer in persons who are continuous users of aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) [4]. These NSAIDs are also effective in reducing colon cancer incidence in animal models. The multiplicity and incidence of cancer were reduced by 30-80% by NSAIDs in the azoxymethane-treated rat and Min/APC mice, animal models of colorectal carcinogenesis [5]. Therefore, the chemopreventive action of NSAIDs might be related to the antiproliferative effect obtained by the inhibition of PG synthesis.

Apoptosis (programmed cell death) inhibition may be an important mechanism by which gastrointestinal cells containing damaged DNA evade normal clearance mechanisms and grow to become invasive tumors. Apoptosis is controlled by a complex interplay between regulatory proteins [6]. Bcl-2, a 26-kDa integral membrane oncoprotein, was the first anti-apoptosis gene product discovered [7]. A popular model of Bcl-2 antiapoptotic function suggests that Bcl-2 actively forms heterodimers with Bax to neutralize the latter's proapoptotic activity [7], and that phosphorylation of Bcl-2 functionally stabilizes the Bcl-2-Bax heterodimerization [8]. Therefore, the Bax/Bcl-2 ratio can function as a controller to modulate cellular fate [9].

We have previously observed that dietary CLA significantly decreased colon tumor incidence and induced apoptosis of the colonic mucosa in 1,2-dimethylhydrazine (DMH)–treated rats [10]. CLA also decreased mucosal levels of PGE_2, thromboxane B_2 (TXB_2), and arachidonate (AA) in a dose-dependent manner. The purpose of the present study was to determine whether CLA inhibits proliferation of colonic epithelium in rats. In addition, we determined whether the decreased $PGE_2$and TXB_2levels in the colonic mucosa of CLA-fed rats are related to changes in COX-2 protein levels. Furthermore, we investigated alterations of Bax/Bcl-2 gene status of colonic mucosa by dietary CLA.

2. Methods and materials

2.1. Experiment 1

2.1.1. Experimental design

All experimental procedures were conducted in compliance with the revised *Guide for the Care and Use of Laboratory animals* published by the National Institutes of Health. A total of 100 male Sprague-Dawley rats 6 weeks of age were purchased from Deahan Animal Experimental Center (Choong-buk, Korea). Upon arrival, they were individually housed in hanging stainless-steel cages on a 12-hour light, 12-hour dark cycle and given Purina rat chow (Ralston-Purina, St. Louis, MO) and tap water *ad libitum*. After 7 days of acclimatization, all rats were randomly assigned to one of two experimental groups. The first group received intramuscular injections with DMH (Aldrich Chemical Co., Milwaukee, WI) at a dose of 15 mg/kg body weight twice per week for 6 weeks to deliver the total dose of 180 mg/kg. The second group received vehicle (0.9% NaCl, pH 6.7). At the same time, each of these two primary groups was randomly divided into two dietary groups and fed either the control (0% CLA) or the experimental (1.0% CLA) diet *ad libitum*. The composition of the control diet was as follows (g/kg): corn starch, 546; casein, 216; L-methionine, 3; cellulose, 37; beef tallow, 120; corn oil, 25; AIN-93 mineral mix, 40; modified AIN-93 vitamin mix, 10; and choline bitartrate, 3. The experimental diet contained the same dietary ingredients as the control diet except that this diet contained 13.6 g CLA-rich oil, 21.4 g corn oil, and 110 g beef tallow as fat sources. CLA-rich oil was synthesized from commercial safflower oil by alkali isomerization as previously described [11], and the fatty acid profile of the oil was determined by gas chromatography as described below. The CLA-rich oil contained 73.6% CLA isomers, and the isomeric composition of the CLA was 50.7% *trans*-10,*cis*-12, 47.2% *cis*-9,*trans*-11 CLA, 1.2% *trans*,*trans*-CLA, and 0.9% other CLA isomers. The amount of CLA-rich oil was calculated to formulate a diet containing 1% (w/w) pure CLA. The fatty acid composition of animal diets is shown in Table 1. Daily food intakes and body weights were determined weekly. The rats were killed 14 weeks after the initiation of the DMH injections and feeding of experimental diets.

Table 1
Fatty acid composition of experimental diet (g/100 g diet)

Fatty Acid	0% CLA	1% CLA
C14:0	0.38	0.35
C14:1	0.14	0.16
C16:0	3.10	2.83
C16:1	0.49	0.45
C17:1	0.12	0.11
C18:0	2.20	2.03
C18:1	5.75	5.38
C18:2*n*-6	1.84	1.68
C18:3*n*-3	0.02	—
CLA*	—	1.09
C20:0	0.14	0.13
C22:6	0.05	0.04
Unknown	0.27	0.25

CLA = conjugated linoleic acid.

* The isomeric composition of the CLA used in this experiment was 47.2% *cis*-9,*trans*-11, 50.7% *trans*-10,*cis*-12, 1.2% *trans*,*trans*, and other CLA isomers.

2.1.2. Immunohistochemistry

Rats were injected intraperitoneally with 5 mg of 5-bromo-2′-dexoyuridine (BrdU, Sigma) per kilogram of body

weight 1 hour before sacrifice. The colon was removed and 1-cm segments of the distal colon were rinsed in phosphate-buffered saline (PBS) and fixed in 10% buffered formalin for immunohistochemistry. The fixed tissues were embedded in paraffin, and paraffin sections (4 μm thickness) were mounted on albumin-coated glass slides and deparaffinized. To detect cell proliferation, BrdU-labeled cells were determined using a monoclonal anti-BrdU antibody and the Zymed BrdU staining kit (Zymed Laboratories, South San Francisco, CA). The incorporation of BrdU into cell DNA, evidenced by a brownish staining, was evaluated in vertically oriented colonic crypts. The number and position of BrdU-positive cells were recorded in addition to the total number of epithelial cells per crypt column. Ten vertically oriented crypt columns were evaluated per animal. Labeling index was calculated as a percentage of BrdU-labeled nuclei divided by the total number of nuclei per crypt column.

For the detection of apoptotic cell death, the terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) method was used, utilizing the In Situ Cell Death Detection Kit, AP (Roche Molecular Biochemical, Mannheim, Germany) as previously described [10]. Ten vertically oriented crypt and villus columns were evaluated per animal. The number of apoptotic cells and total cells were counted. The apoptotic index was calculated as the number of apoptotic nuclei divided by the total number of nuclei per crypt column.

2.1.3. Determination of fatty acid composition of phospholipids and PGE_2, TXB_2, and 1,2-diacylglycerol levels in the colonic mucosa

The colon was removed and the mucosa was scraped from the underlying tissue with a glass slide. Fatty acid profiles of phospholipids and 1,2-diacylglycerol (DAG) levels in colonic mucosa were determined as previously described [10]. Mucosal PGE_2 and TXB_2 were extracted by a solid-phase C-18 cartridge (Alltech Associates, Inc., Deerfield, IL), and the amounts of PGE_2 and TXB_2 were determined using the enzyme immunoassay kits (Cayman, Ann Arbor, MI) as previously described [10].

2.1.4. Western blot analysis

To determine COX-2 protein levels, the mucosa was homogenized and solubilized for 30 min at 4°C with lysis buffer containing 20 mmol/L Hepes, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 100 mmol/L NaF, 10 mmol/L sodium pyrophosphate, and 1 mmol/L Na_3VO_3. The following protease inhibitors were used: 20 μg/mL aprotinin, 10 μg/mL antipain, 10 μg/mL leupeptin, 80 μg/mL benzamidine HCl, and 0.2 mmol/L PMSF. The insoluble material was removed by centrifugation at 13,000 $\times$ g for 10 minutes and protein content was determined using the BCA protein assay kit (Pierce, Rockford, IL). The lysates were resolved on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and transferred onto polyvinylidene fluoride membrane (Millipore). The blots were blocked for 1 h in 1% BSA in TBS-T (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween 20) and incubated for 1 hour with either anti–COX-2 antibody (sc-7951, Santa Cruz Biotechnology, 1:1,000) or β-actin (A5441, Sigma, 1:2,000). The blots were then incubated with antimouse or antirabbit HRP-conjugated antibody (Amersham). Signals were detected by using the enhanced chemiluminescence method using SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL). The abundance of each band was quantified using Image Densitometer (Bio-Rad, Hercules, CA).

2.1.5. Reverse transcriptase–polymerase chain reaction

For the determination of mRNA, the mucosa was scraped quickly and total RNA was isolated using the acid–guanidium thiocyanate–phenol–chloroform method as previously described [12]. The conversion of RNA to cDNA was carried out in a final volume of 20 μL with 1 μg RNA, an oligo-dT primer and avian myeloblastosis virus reverse transcriptase (RT) using the RNA polymerase chain reaction (PCR) kit (version 2.1; Takara Bio Inc., Otsu, Japan). For the PCR reaction, 2 μL of the synthesized cDNA were added to 45 μL of a reaction mixture containing 1$\times$ PCR buffer (20 mmol/L Tris-HCl, 50 mmol/L KCl), 2.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 0.2 μmol/L each of the Bcl-2 (sense: 5′-CAA GCC GGG AGA ACA GGG TA-3′; antisense: 5′-CCC ACC GAA CTC AAA GAA GGC-3′), Bax (sense: 5′-CCG AGA GGT CTT CTT CCG TGT G-3′; antisense: 5′-GCC TCA GCC CAT CTT CTT CCA-3′), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense: 5′-CCC ATC ACC ATC TTC CAG-3′; antisense: 5′-ATG ACC TTG CCC ACA GCC-3′) primers, and 2.5 units of Taq polymerase. After 30 cycles of amplification, the PCR products were analyzed by ethidium bromide–stained agarose gel electrophoresis. The abundance of each transcript was quantified using Image Densitometer. For each combination of primers, the kinetics of PCR amplification were studied, the number of cycles corresponding to plateau were determined, and PCR was performed within the exponential range.

2.2. Experiment 2

The rats (30 rats/group) treated with DMH were continuously fed the assigned diet containing either 0% or 1% CLA *ad libitum*. The animals were sacrificed 30 weeks after the initiation of feeding of the experimental diets; the colon was then removed, opened, and washed in ice-cold PBS. The number of tumors with sizes $\geq$1 mm were counted under a dissecting microscope.

2.3. Statistical analysis

Data were analyzed using Statistical Analysis Systems statistical software package version 6.12 (SAS Institute, Cary, NC). Most data were expressed as means $\pm$ SD.

Table 2
Effects of conjugated linoleic acid (CLA) on 1.2-dimethylhydrazine-induced colon tumors in rats

Diet	Tumor Incidence*	Total Tumor Number	Mean Tumor Number/Rat
0% CLA	13/30	18	0.63
1% CLA	7/30†	10†	0.33†

* Number of tumor-bearing rats/total number of rats per each group.
† $P < 0.05$, different from rats fed the 0% CLA diet.

Two-way analysis of variance (ANOVA), followed by Duncan's multiple range test, was used to analyze the data from Experiment 1. Tumor incidences at the final time point were compared by the χ^2 test, and the total tumor yield between the control and CLA-treated groups was compared by frequency distribution analysis. The level of significance was set at $P < 0.05$.

3. Results

To determine whether CLA lowered colon tumor incidence, rats were injected with DMH and fed the diet containing 1% CLA for 30 weeks. As previously observed [10], tumor incidence and total number of tumors were significantly lower in rats fed the diet containing 1% CLA compared with the 0% CLA diet (Table 2). No differences in food intake or body weight gain were observed in the animals due to either the dietary or carcinogen treatments (data not shown).

The effects of DMH and CLA on mucosal proliferation were analyzed through detection of incorporated BrdU, which was injected 1 hour before the rats were killed. As shown in Table 3, DMH administration resulted in increased labeling index and proliferative zone compared with the corresponding diet group without the carcinogen treatment. The total number of cells per crypt column was not altered either by DMH treatment or by dietary CLA. CLA significantly reduced labeling index and proliferative zone in DMH-treated rats, but the differences between the two vehicle-injected dietary groups did not reach statistical significance ($P > 0.05$).

Table 4
Apoptosis in colonic mucosa of normal and DMH-treated rats fed conjugated linoleic acid (CLA)

Carcinogen	Diet	*N*	Apoptotic index (%)*
Vehicle	0% CLA	6	42.45 ± 7.58^{b}
	1% CLA	5	54.88 ± 6.10^{a}
DMH	0% CLA	6	35.66 ± 2.41^{b}
	1% CLA	6	53.83 ± 5.18^{a}
Two-way ANOVA	Diet effect		<0.0001
	DMH		NS
P values	Interaction		NS

Values are means ± SD; *n* = number of samples. Values in each column with different letters differ, $P < 0.05$.
* Number of apoptotic cells ÷ total number of cells per crypt column × 100.
NS = not significant, $P > 0.05$.

An increase in apoptosis may account for the chemopreventive effect of CLA in DMH-induced colon carcinogenesis. Therefore, we determined the levels of apoptosis in colonic mucosa by the TUNEL method. DMH injection did not change the apoptotic index, whereas CLA significantly increased apoptotic index regardless of whether rats received DMH or vehicle injection (Table 4).

Effects of dietary CLA and DMH on mucosal PGE_2, TXB_2, and DAG are shown in Table 5. Feeding rats the diet containing 1% CLA significantly decreased mucosal levels of PGE_2, TXB_2, and DAG regardless of the carcinogen injection. However, DMH injection did not affect these biomarkers in rats fed either the 0% or 1% CLA diet. There is now an abundance of evidence of an important role for COX-2 in promotion of colorectal cancer development [13]. Therefore, we have determined whether dietary CLA and/or DMH have any effect on COX-2 protein expression. DMH treatment significantly increased COX-2 protein levels in colonic mucosa, although a trend to a decrease in the 1% CLA groups was not statistically significant (Table 6). Because COX-2 levels did not change significantly in animals

Table 3
Cell proliferation in colonic mucosa of normal and DMH-treated rats fed conjugated linoleic acid (CLA)

Carcinogen	Diet	*n*	Crypt Circumference (no. of cells)	Number of Cells/Crypt Column	Labeling index (%)*	Proliferative Zone (%)†
Vehicle	0% CLA	6	26.10 ± 0.81^{a}	28.05 ± 0.39^{a}	5.00 ± 0.33^{bc}	27.33 ± 2.15^{bc}
	1% CLA	3	26.77 ± 0.56^{a}	28.70 ± 0.64^{a}	4.11 ± 0.40^{c}	23.51 ± 1.56^{c}
DMH	0% CLA	6	26.12 ± 0.25^{a}	28.26 ± 0.58^{a}	11.80 ± 0.58^{a}	44.12 ± 1.20^{a}
	1% CLA	6	27.00 ± 0.61^{a}	28.78 ± 0.17^{a}	6.83 ± 0.79^{b}	32.61 ± 2.53^{b}
Two-way ANOVA	Diet effect		0.1951	0.2361	0.0001	0.0005
	DMH effect		0.7714	0.6882	<0.0001	<0.0001
P values	Interaction		0.8596	0.8920	0.0068	0.0593

Values are means ± SD; *n* = number of samples. Values in each column with different letters differ, $P < 0.05$.
* Number of BrdU-labeled cells ÷ total number of cells per crypt column × 100.
† Position of the highest labeled cell ÷ total number of cells per crypt column × 100.

Table 5
Levels of prostaglandin E_2, thromboxane B_2, and 1,2-diacylgycerol in colonic mucosa of rats fed conjugated linoleic acid (CLA)

Carcinogen	Diet	*N*	PGE_2 (ng/mg tissue)	TXB_2 (ng/mg tissue)	1,2-Diacylglycerol (ng/mg tissue)
Vehicle	0% CLA	6	0.52 ± 0.08^{a}	1.91 ± 0.28^{a}	171 ± 36^{a}
	1% CLA	6	0.28 ± 0.14^{b}	1.50 ± 0.15^{bc}	146 ± 16^{b}
DMH	0% CLA	6	0.49 ± 0.17^{a}	1.85 ± 0.41^{ab}	172 ± 19^{a}
	1% CLA	6	0.23 ± 0.44^{b}	1.35 ± 0.21^{c}	144 ± 8^{b}
Two-way ANOVA	Diet effect		0.0004	0.0023	0.0006
	DMH		NS	NS	NS
P values	Interaction		NS	NS	NS

Values are means ± SD; *n* = number of samples. Values in each column with different letters differ, $P < 0.05$.
NS = not significant, $P > 0.05$.

fed 1% CLA, we have determined whether CLA alters fatty acid composition of mucosal phospholipids. As shown in Table 7, CLA was detected in mucosal phospholipids only when rats were fed the diet containing 1% CLA, regardless of whether they received DMH or vehicle. The dietary CLA preparation used in the present study consisted of 51% *trans*-10,*cis*-12 and 47% *cis*-9,*trans*-11 CLA. We did not differentiate the position of conjugated double bonds (*i.e.*, 9,11 versus 10,12). Therefore, the present data could not provide the information on the possible selective incorporation of one CLA isomer over the other into mucosal phospholipids. CLA feeding resulted in marked decrease in AA levels in both DMH-treated and normal rats, indicating that the decrease in the substrate for prostaglandin biosynthesis may be one of the reasons for the decrease in PGE_2 and TXB_2 levels in CLA-fed rats.

To examine whether CLA has any effect on Bax/Bcl-2 gene status of the colonic mucosa, total RNA was isolated from the colonic mucosa and RT-PCR was performed. As shown in Table 8, CLA had no statistically significant effect on Bax mRNA levels in either vehicle- or DMH-injected rats even though there was a trend toward an increase in the 1% CLA groups. CLA tended to decrease Bcl-2 transcripts, which was not statistically significant, whereas DMH injection significantly increased the oncoprotein transcripts. However, DMH decreased but CLA increased the Bax/Bcl-2 ratio.

Table 6
Cyclooxygenase-2 expression in colonic mucosa of normal and DMH-treated rats fed conjugated linoleic acid (CLA)

Carcinogen	Diet	*N*	Cyclooxygenase-2*
Vehicle	0% CLA	4	1.63 ± 0.33^{bc}
	1% CLA	4	1.31 ± 0.57^{c}
DMH	0% CLA	4	3.16 ± 0.90^{a}
	1% CLA	4	2.46 ± 0.73^{ab}
Two-way ANOVA	Diet effect		NS
	DMH		0.0017
P values	Interaction		NS

Values are means ± SD; *n* = number of samples. Values in each column with different letters differ, $P < 0.05$.
* Relative intensity of each band to that of its own β-actin band on Western blots.
NS = not significant, $P > 0.05$.

4. Discussion

Uncontrolled cell proliferation is the hallmark of cancer. It has been shown that CLA reduces the proliferation of a variety of cell types grown in culture [14]. Our *in vitro* studies [15] and others [16] have shown that CLA inhibits proliferation of colon cancer cells. In addition, recently, Kohno et al. [17] have shown that CLA administration lowers the proliferating cell nuclear antigen index in azoxymethane-induced aberrant crypt foci in the colon of rats suggesting CLA inhibition of crypt cell proliferation. The present study provides direct evidence that dietary CLA decreases cell proliferation in the colonic mucosa of rats treated with DMH.

The present results revealed that dietary CLA decreases PGE_2 and TXB_2 levels in colonic mucosa without changing the COX-2 protein levels. These results are consistent with those obtained from *in vitro* studies. We have previously reported that CLA (0-50 μmol/L) inhibited the growth of Caco-2 cells, the human colon adenocarcinoma cell line, in a concentration-dependent manner [18]. However, the same concentrations of CLA did not decrease COX-2 transcript or protein levels of these cells (E.J. Kim and J.H.Y. Park, unpublished results). The present study did not determine the effect of CLA on specific activity of COX-2. Therefore, one cannot rule out the possibility that CLA reduces the specific activity of COX-2. However, the observation that the levels of AA were

Table 7
Fatty acid composition of phospholipids in colonic mucosa of normal and DMH-treated rats fed conjugated linoleic acid (CLA)

Carcinogen	Diet	*n*	CLA*	AA*
Vehicle	0% CLA	4	ND	5.06 ± 1.71^{a}
	1% CLA	4	7.37 ± 0.07	1.35 ± 1.20^{b}
DMH	0% CLA	4	ND	5.56 ± 1.02^{a}
	1% CLA	4	8.34 ± 2.06	1.75 ± 0.77^{b}
Two-way ANOVA	Diet effect			<0.0001
	DMH			NS
P values	Interaction			NS

Values are means ± SD; *n* = number of samples. Values in each column with different letters differ, $P < 0.05$.
* mg Fatty acid/g phospholipid.
ND = not detected; NS = not significant, $P > 0.05$.

Table 8
Bax and Bcl-2 transcripts in colonic mucosa of normal and DMH-treated rats fed conjugated linoleic acid (CLA)

Carcinogen	Diet	*N*	Bax*	Bcl-2*	Bax/Bcl-2 Ratio
Vehicle	0% CLA	6	0.77 ± 0.07	1.09 ± 0.18^{bc}	0.71 ± 0.07^{b}
	1% CLA	6	0.86 ± 0.08	0.95 ± 0.15^{c}	0.91 ± 0.09^{a}
DMH	0% CLA	6	0.71 ± 0.07	1.56 ± 0.31^{a}	0.46 ± 0.05^{c}
	1% CLA	6	0.79 ± 0.07	1.28 ± 0.23^{ab}	0.62 ± 0.06^{b}
Two-way ANOVA	Diet effect		0.1516	0.1882	0.0339
	DMH		0.1363	0.0022	0.0003
P values	Interaction		0.6490	0.8546	0.4484

Values are means ± SD; n = number of samples. Values in each column with different letters differ, $P < 0.05$.
* Relative intensity of each band to that of its own GAPDH band.

markedly decreased in mucosal phospholipids from rats fed 1% CLA indicates that the decrease in eicosanoids were mainly due to the decreased in AA, the substrate for PGE_2. These results and those of others [19,20] indicate that CLA may decrease availability of AA for eicosanoid synthesis via COX, which may be one of the mechanisms responsible for the anticarcinogenic effect of CLA.

Conversion of arachidonate to PGE_2 contributes significantly to tumor growth through the modulation of apoptosis and cellular proliferation [21,22]. The present study confirmed that dietary CLA decreased eicosanoid levels in rat colonic mucosa [10], which may be one reason for the increased apoptosis and decreased cellular proliferation observed in CLA-fed rats. Using human colon cancer cell lines, Tang et al. [23] found that COX-2 overexpression inhibited death receptor 5 expression, upregulated Bcl-2, and attenuated apoptosis induced by tumor necrosis factor–related apoptosis-inducing ligand. In the present study we observed that dietary CLA decreased PGE_2 and TXB_2, and increased the Bax/Bcl-2 transcript ratios in colonic mucosa. With studies using a selective COX-2 inhibitor, SC-58125, Sheng et al. [24] found that treatment of HCA human colon cancer cells with SC-58125 decreased colony formation in monolayer culture and this growth inhibition was reversed by treatment with PGE_2. In addition, PGE_2 inhibited apoptosis caused by this drug and induced Bcl-2 expression in these cells, suggesting that prostanoids may be responsible for maintaining sufficient levels of apoptosis suppressor proteins to ensure cell survival. Much of the evidence accumulated to date suggests that the Bax/Bcl-2 ratio functions as a controller to modulate cellular fate [9]. For example, the results obtained with colorectal cancer cell lines convincingly demonstrated that the ratio of anti-apoptotic Bcl-2 family proteins to Bax plays a key role in the susceptibility of cell to apoptosis induced by NSAIDs. In the present study, dietary CLA increased the Bax/Bcl-2 transcript ratio, suggesting that CLA induces apoptosis of the colonic mucosa by the mechanism similar to those with NSAIDs. It has been also reported that CLA reduced expression of Bcl-2 in premalignant lesions of rat mammary gland [25].

DAG, the nonpolar product of phospolipase C action, is a lipid-soluble second messenger remaining in the plasma membrane, where it activates protein kinase C (PKC) to phosphorylate and thereby modulate the activities of several cellular proteins. PKC has been proposed to play an important role in the etiology of colon cancer by regulating gene expression and a variety of cellular functions including proliferation, differentiation, tumorigenesis, and apoptosis [26,27]. The levels of nuclear DAG fluctuate during the cell cycle [28], and exogenous DAG inhibits the apoptosis triggered by farnesol, ceramide, and daunorubicin, [29–31], suggesting that DAG has important regulatory roles. In the present study, changes in DAG levels (Table 5) were negatively related to those in apoptotic index (Table 4) and were positively related to those in BrdU labeling index in colon mucosa of rats (Table 3). These results are consistent with the idea that DAG is a positive intracellular mitogenic agent [28,32]. Future studies are needed to explore the mechanisms by which CLA regulates DAG production.

In conclusion, we have demonstrated that a diet containing 1% CLA significantly inhibits epithelial cell proliferation and increases the Bax/Bcl-2 transcript ratio in the colonic mucosa of rats treated with DMH. COX-2 levels are not changed, whereas both the concentrations of AA in mucosal phospholipids and the levels of PGE_2 and TXB_2 are decreased in rats fed 1% CLA. We have confirmed that dietary CLA inhibits tumor incidence and increases apoptosis in the colonic mucosa of rats treated with DMH. These results indicate that CLA may decrease mucosal PG levels by modulation of the availability of the substrate for COX but not by modulation of COX-2 protein expression. In addition, the present evidence suggests that the CLA-induced apoptosis of the colonic mucosa may be associated with the increased Bax/Bcl-2 ratio.

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