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Superior Anticarcinogenic Activity of *trans*,*trans*-Conjugated Linoleic Acid in *N*-Methyl-*N*-nitrosourea-Induced Rat Mammary Tumorigenesis

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The anticarcinogenic activity of a mixture of *trans*,*trans*-conjugated linoleic acid (*trans*,*trans*-CLA) was investigated in rat mammary tumorigenesis induced by *N*-methyl-*N*-nitrosourea (MNU), with references to *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA isomers. Female, 7-week-old Sprague−Dawley rats were intraperitoneally injected with MNU (50 mg/kg of body weight) and then subjected to one of five diets (control, 1% *trans*,*trans*-CLA, 1% *cis*-9,*trans*-11-CLA, 1% *trans*-10,*cis*-12-CLA, and 1% linoleic acid; 8 rats/group) for 16 weeks. Food and water were made available *ad libitum*. *trans*,*trans*-CLA significantly ($p < 0.05$) reduced tumor incidence, number, multiplicity, and size and significantly ($p < 0.05$) increased apoptosis, relative to *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA. The molecular mechanism of *trans*,*trans*-CLA was elucidated by measuring apoptosis-related gene products and fatty acid composition in tumors. *trans*,*trans*-CLA led to increases in the p53 protein and Bax protein levels but suppressed the expression of Bcl-2 protein. The activation of caspase-3 led to the cleavage of poly(ADP-ribose) polymerase, which resulted in the execution of apoptosis. In addition, *trans*,*trans*-CLA reduced cytosolic phospholipase A2, cyclooxygenease-2, and peroxisome proliferator-activated receptor γ protein levels. These results suggest that the *trans*,*trans*-CLA inhibits MNU-induced rat mammary tumorigenesis through the induction of apoptosis in conjunction with the reduction of arachidonic acid metabolites and that the efficacy of *trans*,*trans*-CLA is superior to *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA.

KEYWORDS: Conjugated linoleic acid (CLA); *trans*,*trans*-CLA; *N*-methyl-*N*-nitrosourea (MNU); rat mammary tumorigenesis; apoptosis

INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term that refers to a mixture of positional (7,9; 8,10; 9,11; 10,12; 11,13; and 12,14) and geometric (*trans*,*cis*; *cis*,*trans*; *cis*,*cis*; and *trans*,*trans*) isomers of octadecadienoic acid (C18:2) with a conjugated double-bond system (*1*). The predominant CLA isomers in synthetic CLA, synthesized from linoleic acid (LA) by alkaline isomerization, are *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA isomers; small amounts of *trans*-10,*trans*-12-CLA, *trans*-9,*trans*-11-CLA, and other isomers also occur (*2*). Many studies have investigated the beneficial effect of CLA against some types of cancer, atherosclerosis, hypertension, diabetes, and inflammation (*3*).

CLA is a potent anticancer agent in carcinogen-induced carcinogenesis in most animal models and human cancer cell lines tested to date (*4*, *5*). Because CLA contains several isomers, individual CLA isomers (mainly *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA) have been the ones most extensively studied in carcinogen-induced carcinogenesis in experimental animals (*6*−*8*) and cancer cell lines (*9*). To date, it appears that both *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA isomers are similarly active in the inhibition of carcinogen-induced carcinogenesis. For example, diets containing 1% *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA are equally effective in reducing tumors and inducing apoptosis in the colonic mucosa of rats treated with 1,2-dimethylhydrazine (DMH) (*7*), and both *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA isomers significantly decrease *N*-methyl-*N*-nitrosourea (MNU)-induced rat mammary tumors (*6*). However, differential effects between *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA on tumorigenesis have been demonstrated in human cancer cell studies, with the *trans*-10,*cis*-12-CLA isomer exhibiting higher potency against HT-29 colorectal cancer cells (*9*), MG-63 osteosarcoma cells (*10*), and MCF-7 breast cancer cells (*11*), relative to the potency of *cis*-9,*trans*-11-CLA. The differential effects of

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these two CLA isomers on tumorigenesis in animal models and human cancer cell lines have yet to be clarified.

In contrast, little attention has been paid to the inhibitory effects of a mixture of *trans*,*trans*-CLA, containing *trans*-7,*trans*-9-, *trans*-8,*trans*-10-, *trans*-9,*trans*-11-, *trans*-10,*trans*-12-, *trans*-11, *trans*-13-, and *trans*-12,*trans*-14-CLA isomers (*2*) or its individual *trans*,*trans*-CLA isomers on cancer cell lines and chemical-induced animal carcinogenesis. In the current study, we found that *trans*,*trans*-CLA exhibited the greatest potency in growth inhibition of an osteosarcoma cell line, MG-63 (*10*), and a breast cancer cell line, MCF-7 (*11*), relative to *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA. This inhibition was dose-dependent. The *trans*-9,*trans*-11-CLA isomer inhibits the growth of Caco-2 colon cancer cells (*12*) and the development of azoxymethane (AOM)-induced colonic aberrant crypt foci in rats by inducing apoptosis (*13*). Meanwhile, except for one previously published paper by Xasui et al. (*13*) on colon cancer, no reports are available concerning the growth inhibitory effect of *trans*,*trans*-CLA on animal tumorigenesis. Hence, it would be of significance to examine the anticarcinogenic activity of *trans*,*trans*-CLA in carcinogen-induced animal carcinogenesis, especially rat mammary carcinogenesis, as compared to that of *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA.

The purpose of this study was to evaluate the anticarcinogenic activity of *trans*,*trans*-CLA in MNU-induced rat mammary tumorigenesis, relative to *cis*-9,*trans*-11-CLA, *trans*-10,*cis*-12-CLA, and LA. We also examined whether apoptosis mediated by mitochondrial dysfunction is one of the mechanistic anticarcinogenic actions of *trans*,*trans*-CLA.

MATERIALS AND METHODS

Materials. LA (99.0%), MNU, leupeptin, pepstatin, and Mayer's hematoxylin were obtained from Sigma-Aldrich (St. Louis, MO). The *i*-genomic CTB DNA Extraction Mini Kit was obtained from iNtRON Biotechnology (Seongnam, Republic of Korea). Rabbit polyclonal p53, Bcl-2, caspase-3, and Bax antibodies were purchased from Delta Biolabs (Campbell, CA). Rabbit polyclonal caspase-9, cytosolic phospholipase A2 (cPLA2), cyclooxygenase-2 (COX-2), peroxisome proliferator-activated receptor γ (PPARγ), and poly(ADP-ribose) polymerase (PARP) antibodies and anti-rabbit, anti-goat and anti-mouse IgG-horseradish peroxidases were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal caspase-8 antibody was obtained from Oncogene Research Products (San Diego, CA). Monoclonal anti-β-actin was purchased from Sigma-Aldrich. The enhanced chemiluminescence (ECL) Western blotting kit was purchased from Amersham Biosciences (Buckingham, U.K.). Frag EL DNA fragmentation detection kit was purchased from Calbiochem (Burlingame, CA). A mixture of fatty acid methyl ester standards was obtained from Supelco (Bellefonte, PA). All other reagents used were of analytical grade.

Preparation of CLA Isomers. CLA methylester was prepared from LA by alkaline isomerization in propylene glycol, followed by methylation with 1.0 N H_2SO_4/methanol (*14*). The methyl esters of *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA were separated from the CLA methyl esters by low-temperature crystallization (*15*). The *trans*,*trans*-CLA methyl ester was prepared from CLA methyl ester according to the method described previously (*2*). The purity of CLA isomer samples was found to be 94.5% for *cis*-9,*trans*-11-CLA, 99.1% for *trans*-10,*cis*-12-CLA, and 99.0% for *trans*,*trans*-CLA by gas chromatography (GC) (*14*). The isomeric composition of *trans*,*trans*-CLA was found to be 0.7% *trans*-7,*trans*-9-CLA, 8.3% *trans*-8,*trans*-10-CLA, 41.0% *trans*-9,*trans*-11-CLA, 41.6% *trans*-10,*trans*-12-CLA, 7.8% *trans*-11,*trans*-13-CLA, and 0.6% *trans*-12,*trans*-14-CLA, analyzed by silver high-performance liquid chromatography (*1*, *2*).

Animal Experiment. Specific pathogen-free (SPF), female, 7-week-old Sprague-Dawley (SD) rats (Samtako Bio Korea, Osan, Republic of Korea) were housed in polycarbonate cages (1 rat/cage) in a temperature (22 ± 2 °C)- and humidity (55 ± 5%)-controlled SPF facility with a 12 h light/dark cycle system and were fed a basal AIN93G diet (pellet) (Samtako Bio Korea) as a control diet. After 1 week, animals were randomized by body weight and divided into control, 1% *trans*,*trans*-CLA, 1% *cis*-9,*trans*-11-CLA, 1% *trans*-10,*cis*-12-CLA, and 1% LA treatment groups (8 rats for each group). Amounts added as ingredients to each diet were adjusted accordingly by the purity of CLA isomers and LA. Rats were given an intraperitoneal (i.p.) injection of MNU at a single dose of 50 mg/kg of body weight, and 1 day later, they were subjected to diets for 16 weeks. Diet and water were made available *ad libitum*. Starting at the initiation of tumors and continuing thereafter, body weight and food intake were measured weekly. All rats were sacrificed 17 weeks after mammary tumor initiation. Rat care and experimental procedures were in accordance with Gyeongsang National University Animal Ethics Guidelines (GNU-LA-18).

Mammary Tumor Analysis. Tumor weight, size, number, and incidence were recorded from rats sacrificed at the termination of the animal experiment. After all debris had been trimmed off, all of the mammary tumors of each treatment group were longitudinally cut into two pieces and saved for further analysis. One piece from each tumor was fixed immediately in 10% phosphate-buffered formalin (pH 7.3) for histological examination and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay, and the rest of the pieces were kept in a deep freezer maintained at −71 °C until analyzed for biochemical markers.

Histological Examination and TUNEL Assay. Mammary tumors fixed in 10% buffered formalin were embedded in paraffin, followed by the preparation of paraffin sections with a thickness of 4 μm and staining with hematoxylin and eosin (*16*). The mammary tumors were diagnosed and classified as fibroadenoma or adenocacinoma according to the criteria for the classification of rat mammary tumors (*17*). TUNEL assay was applied to detect apoptotic cells in mammary tumors using a Frag EL DNA fragmentation detection kit (*18*). The sections (4 μm thick), adding dioxigenin-labeled nucleotides to the 3′-OH end of the DNA, were mounted in Merckoglas (Merck, Whitehouse Station, NJ) and observed using a model DM6000B microscope (Leica Wetzlar, Germany).

DNA Fragmentation Analysis. Mammary tumor was sliced into a mortar, frozen by the slow addition of liquid nitrogen, disrupted, and homogenized completely. DNA was isolated from the homogenized tissues using the *i*-genomic CTB DNA Extraction Mini Kit (*11*). DNA fragmentation patterns were analyzed by electrophoresis in a 1.2% agarose gel, and the DNA was then treated with ethidium bromide for visualization under UV light.

Western Blotting. Mammary tumors were homogenized in RIPA buffer [150 mM NaCl, 0.1% NP40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), and 50 mM Tris at pH 7.4] containing protease inhibitors, 21 μM leupeptin, and 15 μM pepstatin (*10*). The homogenates were centrifuged at 13 000 rpm for 5 min at 4 °C, and the supernatant was collected for protein samples. The protein content was determined by the Bradford protein assay protocol. Western blotting for p53, Bax, Bcl-2, caspase-3, PARP, PPARγ, cPLA2, COX-2, caspase-9, and caspase-8 proteins were performed according to previously described methods (*10*, *11*). Bound antibodies were detected under UV light with the assistance of an ECL Western blotting detection kit. The relative protein levels were determined using an Eastman Kodak model Gel Logic 100 Imaging System (Eastman Kodak, Rochester, NY).

Determination of Phospholipids. Total mammary tumor lipid was extracted, and the phospholipid fraction from total lipids was separated using a silica Maxi-Clean cartridge as previously described (*19*). The acquired phospholipid was methylated with 20% tetramethylguanidine (TMG) in methanol (100 °C, 10 min), followed by an additional reaction with 1.0 N H_2SO_4/methanol (55 °C, 5 min) (*2*). Fatty acid methyl esters were analyzed by GC (Hewlett-Packard 5890) equipped with a flame ionization detector (FID) and a fused silica capillary column Supelcowax-10 (60 m × 0.32 mm inner diameter, 25 μm film thickness). The oven temperature was increased from 180 to 200 °C at a rate of 2 °C/min and then held for 30 min. Injection port and detector temperatures were 240 and 260 °C, respectively. Nitrogen (99.9%) was used as a carrier gas with a flow rate of 2 mL/min. The fatty acids of the samples were identified through the comparison to the relative retention time of the standards.

Statistical Analysis. The results are presented as mean ± standard deviation (SD). Statistical analysis was carried out using analysis of variance (ANOVA), followed by Duncan's multiple range test. Mean differences at $p < 0.05$ were considered to indicate statistical significance.

RESULTS

Inhibition of MNU-Induced SD Rat Mammary Tumorigenesis by *trans*,*trans*-CLA. Table 1 shows the effect of dietary *trans*,*trans*-CLA on the tumor incidence, number, multiplicity, and average tumor size of SD rats treated with MNU, with references to *cis*-9, *trans*-11-CLA, *trans*-10,*cis*-12-CLA, and LA. The efficacy of *trans*, *trans*-CLA on the inhibition of tumorigenesis was superior to the other CLA isomers. Control rats given MNU alone had 100% tumor incidence. Tumor incidence in *trans*,*trans*-CLA-treated rats was significantly ($p < 0.05$) reduced, as compared to that of *trans*-10,*cis*-12-CLA-, *cis*-9,*trans*-11-CLA-, and LA-treated rats. Similar results were obtained from the total tumor number, tumor multiplicity, and tumor size. Tumor incidence of rats treated with LA was significantly ($p < 0.05$) reduced, relative to control rats. This could be, in part, due to LA metabolite, such as (±)-13-hydroxy-10-oxo-*trans*-11-octadecenoid acid (13-HOA) acting as a anti-promoter in mice (*20*), although LA acts as a tumor promoter in rats (*21*). Further research should be performed to clarify this event.

Table 1. Effect of *trans*,*trans*-CLA on the Incidence, Total Number, Multiplicity, and Size of Tumors in SD Rats Induced by MNU

treatment[a]	incidence (%)[b]	total tumor[c]	multiplicity (tumor/rat)[d]	tumor size (cm)[e]
control	100 a	30	5.0 ± 0.8[f] a	4.5 ± 0.8 a
1% LA	83 b	27	4.5 ± 0.8 a	3.9 ± 0.5 a
1% *cis*-9,*trans*-11-CLA	67 c	18	3.0 ± 0.6 b	2.8 ± 0.4 b
1% *trans*-10,*cis*-12-CLA	50 d	16	2.7 ± 0.4 b	2.4 ± 0.6 b
1% *trans*,*trans*-CLA	33 e	12	2.0 ± 0.3 c	1.8 ± 0.3 c

[a] Rats were injected, i.p., with a single dose of MNU (50 mg/kg of body weight) and, 1 day later, subjected to one of the following diets for 16 weeks: control, 1% LA, 1% *cis*-9,*trans*-11-CLA, 1% *trans*-10,*cis*-12-CLA, and 1% *trans*,*trans*-CLA. [b] Percentages of tumor-bearing rats against eight rats in a given treatment group. Tumor incidence with different letters is significantly different at $p < 0.05$ by the χ^2 test. [c] Total number of tumors induced in a given treatment group with eight rats. [d] Number of tumors per tumor-bearing rat. [e] Average tumor size ≥0.3 mm in diameter. [f] Mean ± SD ($n = 8$). Means with different letters are significantly different at $p < 0.05$ by Duncan's multiple range tests.

As seen in **Figure 1**, *trans*,*trans*-CLA-treated rats maintained a higher body weight than *cis*-9,*trans*-11-CLA-, *trans*-10,*cis*-12-CLA-, and LA-treated rats from week 6 after MNU treatment, which might be associated with the enhanced food intake levels. The body weight of *trans*,*trans*-CLA-treated rats at week 16 was maintained significantly ($p < 0.05$) higher than other CLA-treated rats. The ability of *trans*,*trans*-CLA to maintain the appetite and the body weight may be related to a possible anti-cachectic action of this compound (*22*).

Histology of Tumors from SD Rats Treated with *trans*,*trans*-CLA. Figure 2 shows the results of microscopic analysis of tumors stained with hematoxylin and eosin (*17*). The tumors in control rats were all adenocarcinomas. The lesions were rather cellular and consisted of a proliferation of epithelial and stromal components; the epithelial elements ranged from reactive to malignant in nature. The majority of the tumors was adenomatous and exhibited features of florid sclerosing adenosis mixed with low-grade ductal carcinoma *in situ*. Focal areas exhibited features

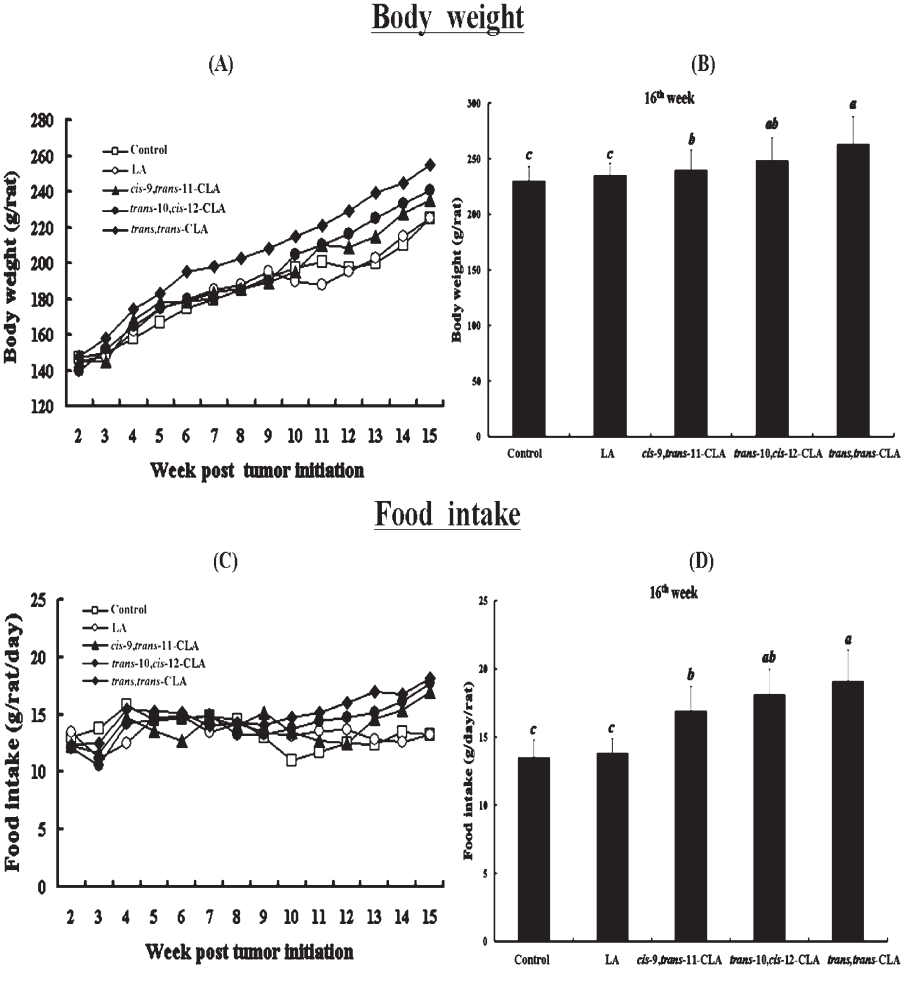

Figure 1. Effect of dietary *trans*,*trans*-CLA on body weight and food intake of rats treated with MNU. Data are presented as mean ± SD of eight independent rats. Means with different letters are significantly different at $p < 0.05$ by Duncan's multiple range tests.

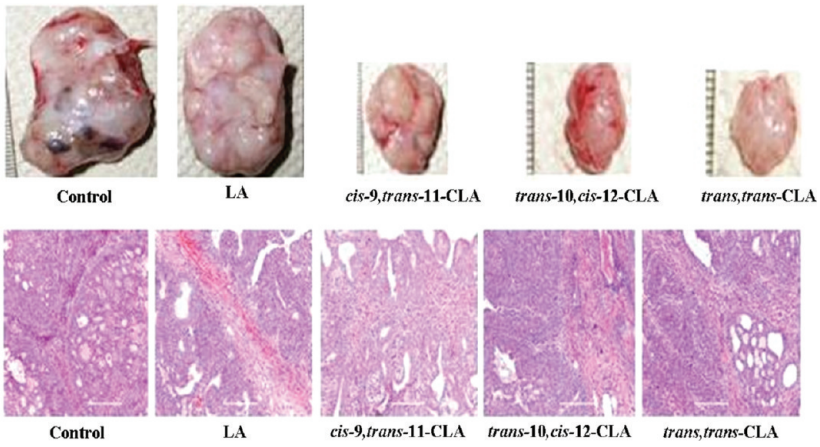

Figure 2. Histochemical analysis of MNU-induced mammary tumors from rats treated with *trans,trans*-CLA. Tumor tissues were stained with hematoxylin and eosin.

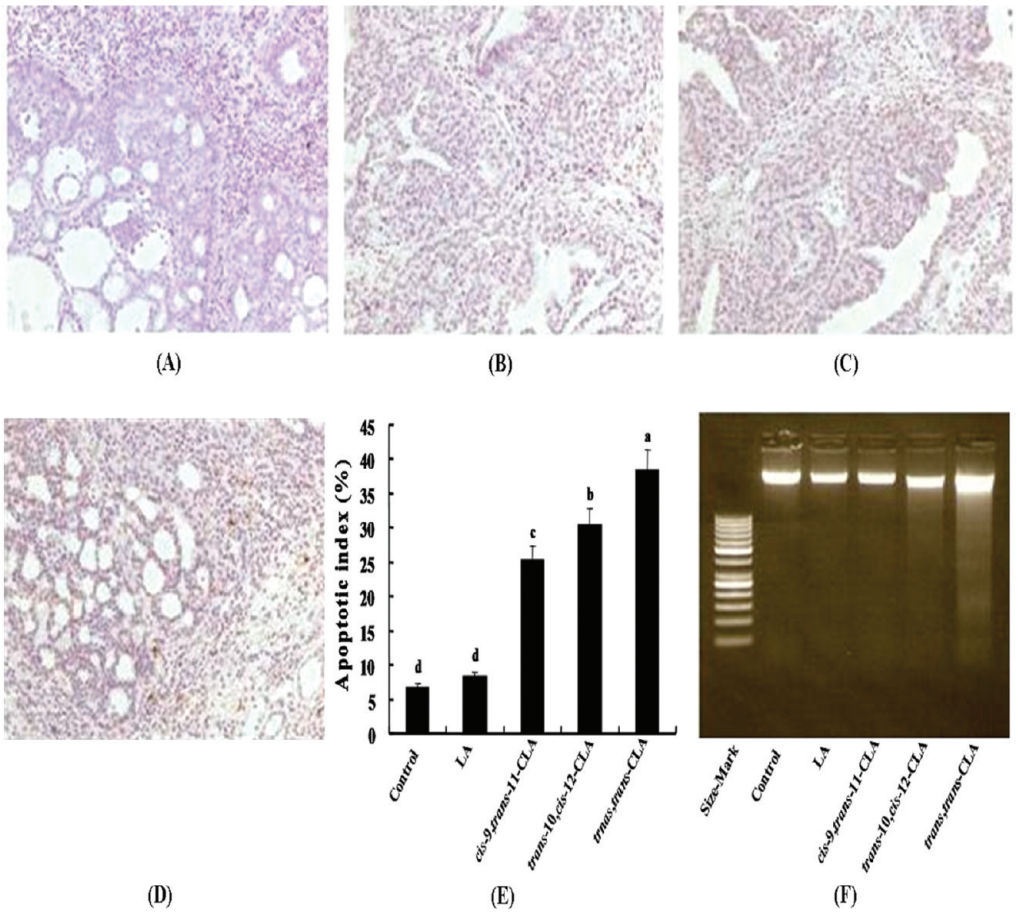

Figure 3. Induction of apoptosis in MNU-induced rat mammary tumors by *trans,trans*-CLA. Apoptosis of tumors was assayed by TUNEL (A−E) and DNA fragmentation (F) assays. (A−D) Control, *cis*-9,*trans*-11-CLA, *trans*-10,*cis*-12-CLA, and *trans,trans*-CLA treatments, respectively. The apoptotic cell index is shown in E. The size markers used are indicated in F. Means with different letters are significantly different at $p < 0.05$ by Duncan's multiple range tests.

characteristic of adenocarcinoma, including increased mitotic indices, atypical mitotic figures, moderate-to-severe cytologic atypia, coagulative tumor cell necrosis, and jagged infiltrating margins. The lesions were well-circumscribed in some areas and were associated with a brisk host response composed of reactive stromal cells and a mixed inflammatory infiltrate. Angiogenesis was much more prominent in these lesions than in the adenomas of the supplemented rats. In contrast, tumors from *trans,trans*-CLA-treated rats contained epithelial cells that appeared mildly to moderately cytologically atypical and had low mitotic indices. Lesions were well-circumscribed and exhibited prominent papillary architecture. In the tumors from rats treated with *cis*-9, *trans*-11-CLA, *trans*-10,*cis*-12-CLA, and LA, lesions were moderately cellular and consisted of epithelial and stromal components. The overall cytoarchitectural features were indicative of a fibroepithelial lesion, such as a fibroadenoma. The stromal and vascular proliferation was much less than that seen in adenocarcinoma tumors of control rats. These results suggest that the anticarcinogenic potential of *trans,trans*-CLA was superior to other CLA compounds in rat mammary turmorigenesis induced by MNU.

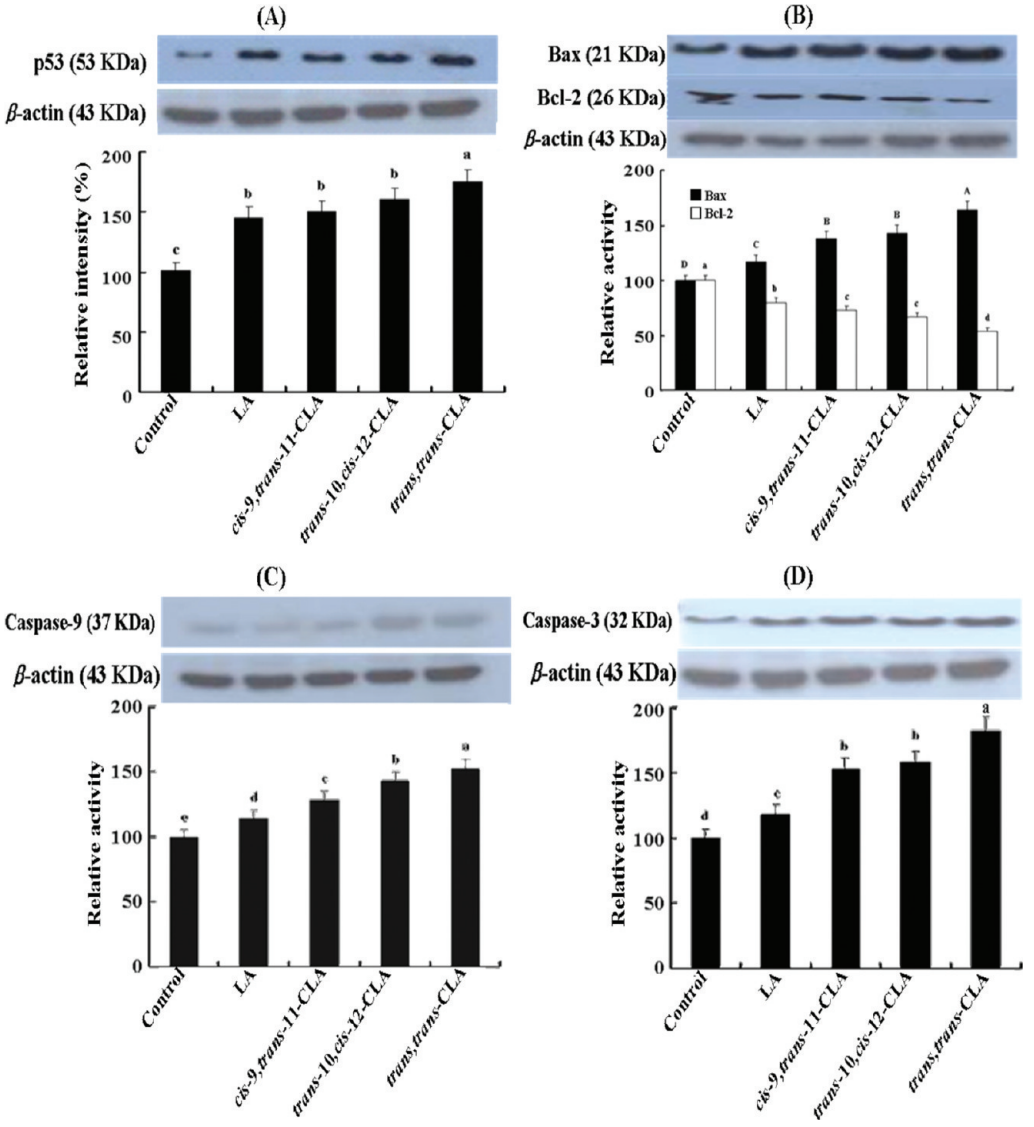

Figure 4. Gene expressions and enzyme activations related to apoptosis in MNU-induced rat mammary tumors by *trans*,*trans*-CLA. The band intensities were quantified relative to the control. Bars indicate means ± SD ($n = 3$). (A−D) p53, Bax and Bcl-2, caspase-9, and caspase-3, respectively. Means with different letters are significantly different at $p < 0.05$ by Duncan's multiple range tests.

Induction of Apoptosis in Tumors from SD Rats Treated with *trans*,*trans*-CLA. Apoptosis was determined by TUNEL (panels **A**−**E** of **Figure 3**) and DNA fragmentation (**Figure 3F**) assays, which are considered hallmarks of apoptosis, for mammary tumor tissues from rats treated with *trans*,*trans*-CLA, with references to *cis*-9,*trans*-11-CLA, *trans*-10,*cis*-12-CLA, and LA. TUNEL analysis showed more frequent occurrence of darkly stained TUNEL-positive nuclei in the mammary epithelium of *trans*,*trans*-CLA-treated rats than in the mammary epithelia of the control and *cis*-9,*trans*-11-CLA-, and *trans*-10,*cis*-12-CLA-treated rats. The apoptotic cell indices were significantly higher ($p < 0.05$) in the tumors from *trans*,*trans*-CLA-treated rats than in the tumors from *cis*-9,*trans*-11-CLA- and *trans*-10,*cis*-12-CLA-treated rats, although *cis*-9,*trans*-11-CLA- and *trans*-10,*cis*-12-CLA-treated rats maintained significantly higher ($p < 0.05$) indices in the tumors than LA-treated or control rats (**Figure 3E**).

Mammary tumors from rats treated with *trans*,*trans*-CLA produced a distinct smear of DNA fragments (**Figure 3F**), a typical characteristic of cells undergoing apoptosis. The *trans*-10,*cis*-12-CLA also produced a characteristic smearing but at a lesser extent than *trans*,*trans*-CLA. No or little smearing was seen in tumors from the control, *cis*-9,*trans*-11-CLA-, and LA-treated rats. Given the results from TUNEL and DNA fragmentation assays, the induction of apoptosis by *trans*,*trans*-CLA was greater than that of *cis*-9,*trans*-11-CLA, *trans*-10,*cis*-12-CLA, or LA.

Expression of Apoptotic-Related Proteins from SD Rats Treated with *trans*,*trans*-CLA. The expression of p53, Bax, and Bcl-2 genes was assessed by Western blotting to establish the role of apoptosis in MNU-induced mammary tumorigenesis (**Figure 4**). The p53 tumor suppressor protein was significantly increased ($p < 0.05$) in tumors from *trans*,*trans*-CLA-treated rats, relative to the protein levels in tumors from *cis*-9,*trans*-11-CLA- and *trans*-10,*cis*-12-CLA-treated rats. *trans*,*trans*-CLA treatment induced a significantly higher level of expression of Bax in the tumors ($p < 0.05$), as compared to the treatment with *cis*-9,*trans*-11-CLA, *trans*-10,*cis*-12-CLA, and LA. In contrast to p53 and Bax proteins, the expression of anti-apoptotic Bcl-2 was significantly reduced in *trans*,*trans*-CLA-treated mammary tumors ($p < 0.05$), relative to the expression levels in the *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA groups. Consequently, *trans*,*trans*-CLA-induced apoptosis in MNU-induced mammary tumorigenesis was associated with a reduced Bcl-2 protein level and an increased Bax protein level.

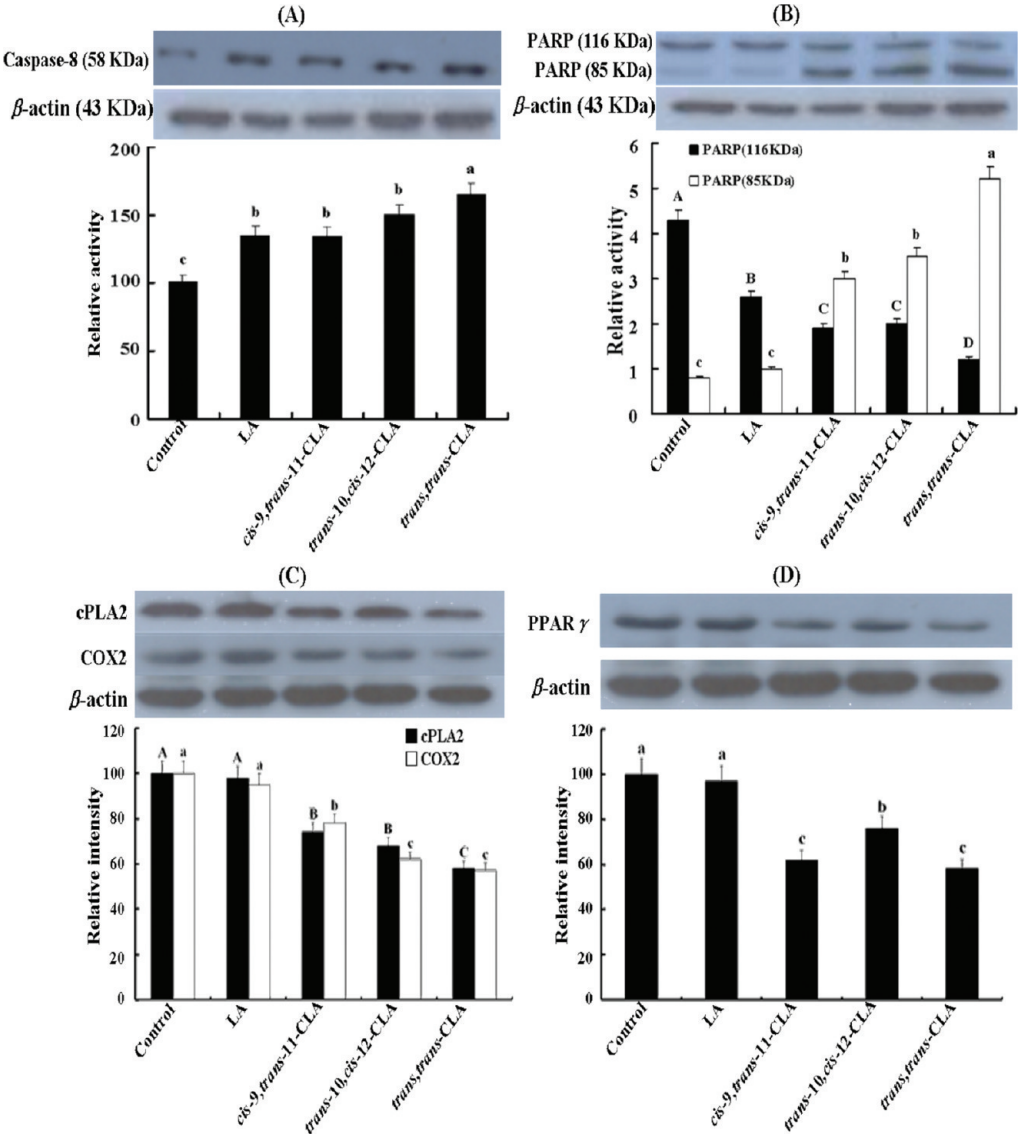

Figure 5. Gene expressions and enzyme activations related to apoptosis and/or AA metabolism in MNU-induced rat mammary tumors by *trans*,*trans*-CLA. The band intensities were quantified relative to the control. Bars indicate means ± SD ($n = 3$). (A−D) Caspase-8, PARP, cPLA2, and PPARγ, respectively. Means with different letters are significantly different at $p < 0.05$ by Duncan's multiple range tests.

To determine whether caspase activation is a required signaling event for apoptosis and whether increases in caspase activity coincided with apoptotic cell death, the protein levels of caspase-3, caspase-9, and caspase-8 were measured in tumors from rats treated with *trans*,*trans*-CLA. *trans*,*trans*-CLA treatment significantly elevated caspase-9 and caspase-3 protein levels ($p < 0.05$), relative to the levels observed following *cis*-9,*trans*-11-CLA, *trans*-10,*cis*-12-CLA, and LA treatments (**Figure 4**). The protein levels of caspase-9 and caspase-3 in the tumors of rats treated with *trans*,*trans*-CLA were 1.5- and 1.8-fold higher than those seen in control rats, respectively. A similar result with the caspase-8 protein level was seen in tumors treated with *trans*,*trans*-CLA (**Figure 5**). The degradation of PARP, an intrinsic substrate for caspase-3 that is normally cleaved during apoptosis, in tumors from rats treated with the *trans*,*trans*-CLA isomer was evident (**Figure 5**). A 85 kDa product was found in these tumors, together with the concomitant loss of the 116 kDa full-length PARP. The relative breakdown ratio of the 85 kDa product to 116 kDa was 0.2 in tumors from control rats but was elevated to 0.7 in tumors from rats treated with *trans*,*trans*-CLA, 1.6 in tumors from rats treated with *cis*-9,*trans*-11-CLA, and 1.7 in tumors from rats treated with *trans*-10,*cis*-12-CLA. Overall, the results indicated that *trans*,*trans*-CLA-induced apoptosis in MNU-induced mammary tumorigenesis was associated with an increased level of p53 and Bax proteins, reduced Bcl-2 protein level, and activation of caspase-8 and caspase-3, thus leading to PARP cleavage.

Reduction of cPLA2, COX-2, and PPARγ Expressions and Composition of Major Fatty Acids in Tumors from SD Rats by *trans*,*trans*-CLA. Arachidonic acid (AA) metabolites are closely related to carcinogenesis in animal models (*23*). Appropriately, we measured the expression of the cPLA2, COX-2, and PPARγ genes, which are related to AA metabolism, in MNU-induced rat mammary tumors treated with *trans*,*trans*-CLA (**Figure 5**). The expression of cPLA2 protein was significantly ($p < 0.05$) lowered in rat mammary tumors treated with *trans*,*trans*-CLA, relative to that of control, *cis*-9,*trans*-11-CLA, and *trans*-10,*cis*-12-CLA treatments. Similar results were seen for COX-2 protein expression. The reduction efficacy of *trans*,*trans*-CLA on PPARγ protein expression was superior to that of *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA but was not significant. LA treatment was not effective on the reduction of cPLA2, COX-2, and PPARγ protein expressions.

Table 2. Composition of Major Fatty Acids in the Phospholipid Fractions of Mammary Tumors from SD Rats Treated with MNU

		treatment[a]			
fatty acid[b]	control	1% LA	1% *cis*-9,*trans*-11-CLA	1% *trans*-10,*cis*-12-CLA	1% *trans*,*trans*-CLA
palmitic (C16:0)	24.6[c] b	24.4 b	28.7 a	30.4 a	28.0 a
stearic (C18:0)	14.8 a	12.1 b	11.2 b	15.2 a	15.4 a
oleic (C18:1)	32.1 b	34.0 a	34.5 a	26.2 c	27.7 c
linoleic (C18:2)	13.8 b	17.3 a	14.6 b	9.3 c	9.4 c
α-linolenic (C18:3)	3.7 a	2.7 a	1.4 a	2.7 a	1.6 b
arachidonic (C20:4)	11.0 b	8.6 c	8.2 c	13.6 b	16.3 a
cis-9,*trans*-11-CLA	d	d	1.1 a	0.5 b	d
trans-10,*cis*-12-CLA	d	d	0.4 b	2.1 a	d
trans,*trans*-CLA	d	d	d	d	1.6

[a] Rats were injected ip with a single dose of MNU (50 mg/kg of body weight) and, 1 day later, subjected to one of the following diets for 16 weeks: control, 1% LA, 1% *cis*-9,*trans*-11-CLA, 1% *trans*-10,*cis*-12-CLA, and 1% *trans*,*trans*-CLA. [b] The composition of fatty acids was calculated by the ratio of the chromatogram peak area of a given fatty acid to total peak areas of interesting fatty acids obtained from GC. [c] Mean ± SD ($n = 3$). SD is less than 5% of the mean value. Means with different letters in the same row are significantly different at $p < 0.05$ by Duncan's multiple range test. [d] Not detectable.

Table 2 shows major fatty acid composition in the membrane phospholipid fraction from tumors of rats treated with *trans*, *trans*-CLA. *trans*,*trans*-CLA treatment significantly ($p < 0.05$) increased the composition of AA in membrane phospholipids, as compared to control and other treatments. Meanwhile, *trans*-10, *cis*-12-CLA also significantly ($p < 0.05$) increased the composition of AA, but *cis*-9,*trans*-11-CLA and LA treatments rather decreased relative to the control treatment. The increasing efficacy of *trans*,*trans*-CLA on AA composition was significantly ($p < 0.05$) higher than that of other CLA isomers. The composition of palmitic acid by *trans*,*trans*-CLA, *cis*-9,*trans*-1 1-CLA, and *trans*-10,*cis*-12-CLA treatments was significantly ($p < 0.05$) elevated, as compared to that of control and LA treatments, but no significance was observed among that of CLA isomer treatments.

DISCUSSION

The present study clearly revealed that *trans*,*trans*-CLA inhibits MNU-induced mammary carcinogenesis in rats. The inhibitory efficacy of *trans*,*trans*-CLA was superior to that of the equally effective *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA and could be, in part, associated with the induction of apoptosis of tumor cells through antitumor promoter action. No direct comparison data for the anticarcinogenicity of *trans*,*trans*-CLA with *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA on chemical-induced mammary carcinogenesis in rats is available in the published literature, but this finding is in agreement with the inhibitory efficacy of *trans*,*trans*-CLA and *trans*-9,*trans*-11-CLA on human cancer cell lines investigated thus far. *trans*,*trans*-CLA displays a stronger anti-proliferative activity in both MG-63 osteosarcoma cells (*10*) and MCF-7 breast cancer cells (*11*) by inducing apoptosis, relative to that of *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA, which exhibit a similar inhibitory effect. *trans*-9,*trans*-11-CLA also has been demonstrated to exhibit an even higher efficiency than both *cis*-9,*trans*-11-CLA and *trans*-10, *cis*-12-CLA in inducing apoptosis in human colon cancer cells (*12*). The collective data strongly suggests that the minor *trans*,*trans*-CLA isomer should be considered, because it could exert even more powerful antitumor effects than those observed with other CLA isomers.

The anticarcinogenic action of *trans*,*trans*-CLA isomers is not well-defined, but it may be related to its chemical structure, because the anticarcinogenic efficacy of *trans*,*trans*-CLA is superior to *trans*-10,*cis*-12-CLA and *cis*-9,*trans*-11-CLA in various cancer cell lines (*10*, *11*). Furthermore, it is evident that the anticarcinogenic action of *trans*-10,*cis*-12-CLA is even greater than that of *cis*-9,*trans*-11-CLA in chemical-induced rat mammary tumorigenesis (*8*) and various types of cancer cell lines (*9*−*12*), whereas LA acts as a promoter (*22*). Likewise, in this study, the potency of anticarcinogenic activities were directly related to the configuration at the double bonds of CLA isomers; *trans*,*trans*-CLA is a straight chain that resembles stearic acid (C18:0) and shows the strongest anticarcinogenic activity, whereas *cis*-9,*trans*-11 CLA resembles LA and shows the least activity. This hypothesis of the structural configuration of *trans*, *trans*-CLA may also suggest that the anticarcinogenic action of the *trans*,*trans*-CLA in MNU-induced tumorigenesis occurs through the modulation of the expressions of apoptosis-related genes.

The molecular mechanism by which *trans*,*trans*-CLA inhibits MNU-induced tumorigenesis in rats is not yet well-understood. However, the inhibition by *trans*,*trans*-CLA can be explained, in part, by the alteration in the caspase-dependent apoptosis caused by Bax, Bcl-2, caspase-9, and caspase-3 (**Figures 4** and **5**). These results are in agreement with the results shown in some human cancer cell lines (*24*). In addition, *trans*,*trans*-CLA induced the expression of the caspase-8 protein (**Figure 5**), which indicates that caspase-8 is involved in the induction of apoptosis in tumor cells from rats treated with *trans*,*trans*-CLA through the cleavage of cytosolic, cytoskeletal, and nuclear proteins, as well as DNA (*25*), and the release of cytochrome *c* from mitochondria (*26*). Such extrinsic pathway events at an early stage should be further examined at time intervals.

Although upregulation of p53, reciprocal regulation of Bax and Bcl-2, and activation of caspase-8 and caspase-3 might be sufficient to shift the balance toward apoptosis in MNU-induced rat mammary cacrcinogenesis, AA and its metabolites are also associated with apoptosis. *trans*,*trans*-CLA treatment increased the AA composition in the membrane phospholipids (**Table 2**) and suppressed the expression of cPLA2 and COX-2 proteins (**Figure 5**), relative to those of *trans*-10,*cis*-12-CLA and *cis*-9, *trans*-11-CLA. These results may indicate the reduced biosynthesis of prostaglandin E2 (PGE2) from AA, which is reduced in concentration in cytosol, via the COX-2 and prostaglandin H synthase pathways, because the *cis*-9,*trans*-11-CLA and *trans*-10, *cis*-12-CLA isomers inhibit the formation of AA and PGE2 in the mucosal cells of 1,2-dimethylhydrazine-induced rats (*8*) and in cancer cells (*27*). These results of increased AA content in the membrane and lowered cPLA2 and COX2 by *trans*,*trans*-CLA, which might reduce the AA concentration and PGE2 in cytosol, are in agreement with results that the positive association of AA with apoptosis was proven only for free AA in cytosol or exogenously added AA in the culture media by Monjazeb et al. (*23*). There is growing evidence that AA and PGE2 exert

anti-apoptotic effects in various cancer cells and chemical-induced carcinogenesis in animal modes (*28*), confirming the data obtained in the present study. Because cPLA2 is cleaved by caspase-3 and/or a related caspase in HeLa cells undergoing apoptosis (*29*), the activated caspase-3 in the tumors treated with *trans*,*trans*-CLA might be involved in the cleavage of cPLA2, leading to a lower AA content and COX-2 protein level in *trans*, *trans*-CLA-treated tumors. These suggest that *trans*,*trans*-CLA induced apoptosis of tumor cells through the reduction of cPLA2 and COX-2 activities, which act as tumor promoters.

The activation of PPARγ by ligands leads to either inhibition of cell proliferation or induction of apoptosis in human breast tumors and other cancers and human breast cancer cell lines as well (*30*). Of the PPARγ ligands, CLA isomers display high affinity for and are an activator of PPARγ (*31*). Hence, the anticarcinogenic activity of CLA is partially mediated by PPARγ activation in susceptible tumors. However, in the present study, PPARγ expression was significantly ($p < 0.05$) reduced by *trans*, *trans*-CLA and other CLA isomers, relative to that of control and LA treatment, with no significant difference between CLA isomers (**Figure 5**). This inverse relation of PPARγ data shown in the present study and in the literature might be partly due to the fact that endogenous ligands for PPARγ are limited in the tumors treated with *trans*,*trans*-CLA, because COX-2 products, such as 15-deoxy-D12,14-PGJ2, are probably the most potent endogenous PPARγ ligands (*32*). The suppressed PPARγ and COX-2 proteins by *trans*,*trans*-CLA might also be involved in the molecular mechanism of anticarcinogenicity of *trans*,*trans*-CLA, including *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA isomers. Further studies should clarify this issue.

Because *trans*,*trans*-CLA acts as an anti-promoter in the present study, it suppressed tumor promotion by reducing cPLA2 and COX-2 (**Figure 5C**), which act as tumor promoters, and by probably blocking endogenous hormones from acting as tumor promoters. It is also another mechanism that *trans*,*trans*-CLA either prevented tumor promotion from inhibiting gap junction function or increased gap junction function in tumor cells (*33*,*34*). Further studies should be performed to clarify such events.

In conclusion, dietary *trans*,*trans*-CLA inhibits mammary carcinogenesis in MNU-induced SD rats by inducing apoptosis through the reciprocal expressions of Bcl-2 and Bax and the caspase pathway and by reducing eicosanoids through the cPLA2 pathway. The inhibitory action of *trans*,*trans*-CLA is superior to those of the *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA isomers. The precise mechanistic action and signaling event involved in *trans*,*trans*-CLA-induced apoptosis in mammary tumors remains to be determined. We strongly suggest that the *trans*,*trans*-CLA isomer should be considered in tumor therapy because of its superior antitumor effects, as compared to the *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA isomers.

LITERATURE CITED

(1) Sehat, N; Kramer, J. K. G.; Mossoba, M. M.; Yurawecz, M. P.; Roach, J. A. G.; Eulitz, K.; Morehouse, K. M.; Ku, Y. Identification of conjugated linoleic acid isomers in cheese by gas chromatography, silver ion high performance liquid chromatography and mass spectral reconstructed ion profiles-Comparison of chromatographic elution sequences. *Lipids* **1998**, *33*, 963–971.

(2) Kim, S. J.; Park, S. J.; Kim, J. K.; Kim, J. H.; Shim, K. H.; Park, C. G.; Kim, J. O.; Ha, Y. L. Preparation of a *t*,*t* conjugated linoleic acid methylester (CLA-Me) isomers mixture from synthetic CLA by methylation with BF_3/methanol. *J. Agric. Food Chem.* **2003**, *51*, 3208–3214.

(3) Serini, S.; Piccioni, E.; Merendino, N.; Calviello, G. Dietary polyunsaturated fatty acids as inducers of apoptosis: Implication for cancer. *Apoptosis* **2009**, *14*, 135–152.

(4) Ha, Y. L.; Storkson, J. M.; Pariza, M. W. Inhibition of benzo[*a*]-pyrene induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. *Cancer Res.* **1990**, *50*, 1097–1101.

(5) Miglietta, A.; Bozzo, F.; Bocca, C.; Gabriel, L.; Trombetta, A.; Belotti, S.; Canuto, R. A. Conjugated linoleic acid induces apoptosis in MDA-MB-231 breast cancer cells through ERK/MAPK signalling and mitochondrial pathway. *Cancer Lett.* **2006**, *234*, 149–157.

(6) Hubbard, N. E.; Lim, D.; Erickson, K. L. Effect of separate conjugated linoleic acid isomers on murine mammary tumorigenesis. *Cancer Lett.* **2003**, *190*, 13–19.

(7) Park, H. S.; Chun, C. S.; Kim, S.; Ha, Y. L.; Park, J. H. Dietary *trans*-10,*cis*-12 and *cis*-9,*trans*-11 conjugated linoleic acids induce apoptosis in the colonic mucosa of rats treated with 1,2-dimethylhydrazine. *J. Med. Food* **2006**, *9*, 22–27.

(8) Meng, X.; Shoemaker, S. F.; McGee, S. O.; Ip, M. M. *t*10,*c*12-Conjugated linoleic acid stimulates mammary tumor progression in Her2/ErbB2 mice through activation of both proliferative and survival pathways. *Carcinogenesis* **2008**, *29*, 1013–1021.

(9) Cho, H. J.; Kim, W. K.; Jung, J. I.; Kim, E. J.; Lim, S. S.; Kwon, D. Y.; Park, J. H. Y. *trans*-10,*cis*-12, not *cis*-9,*trans*-11, conjugated linoleic acid decreases ErbB3 expression in HT-29 human colon cancer cells. *World J. Gastroenterol.* **2005**, *11*, 5142–5150.

(10) Kim, Y. S.; Cerbo, R. M.; Hah, C. K.; Bahn, K. N.; Kim, J. O.; Ha, Y. L. Growth inhibition of osteosarcoma cell MG-63 by a mixture of *trans*,*trans* conjugated linoleic acid isomers: Possible mechanistic actions. *J. Food Sci.* **2008**, *73*, 247–255.

(11) Islam, M. A.; Kim, Y. S.; Jang, W. J.; Lee, S. M.; Kim, H. G.; Kim, S. Y.; Kim, J. O.; Ha, Y. L. A mixture of *trans*,*trans* conjugated linoleic acid induces apoptosis in MCF-7 human breast cancer cells with reciprocal expression of Bax and Bcl-2. *J. Agric. Food Chem.* **2008**, *56*, 5970–5976.

(12) Beppu, F.; Hosokawa, M.; Tanaka, L.; Kohno, H.; Tanaka, T.; Miyashita, K. Potent inhibitory effect of *trans*-9,*trans*-11 isomer of conjugated linoleic acid on the growth of human colon cancer cells. *J. Nutr. Biochem.* **2006**, *17*, 830–836.

(13) Yasui, Y.; Suzuki, R.; Kohno, H.; Miyamoto, S.; Beppu, F.; Hosokawa, M.; Miyashita, K.; Tanaka, T. 9-*trans*,11-*trans* conjugated linoleic acid inhibits the development of azoxymethane-induced colonic aberrant crypt foci in rats. *Nutr. Cancer* **2007**, *59*, 82–91.

(14) Park, S. J.; Park, C. W.; Kim, S. J.; Kim, J. K.; Kim, Y. R.; Park, K. A.; Kim, J. O.; Ha, Y. L. Methylation methods for the quantitative analysis of conjugated linoleic acid (CLA) isomers in various lipid samples. *J. Agric. Food Chem.* **2002**, *50*, 989–996.

(15) Kim, S. J.; Park, K. A.; Park, J. H. Y.; Kim, J. O.; Ha, Y. L. Preparation of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 conjugated linoleic acid (CLA) isomers from synthetic CLA. *J. Food Sci. Nutr.* **2000**, *5*, 70–74.

(16) Wong, P.; Coulombe, P. A. Loss of keratin 6 (K6) proteins reveals a function for intermediate filaments during wound repair. *J. Cell Biol.* **2003**, *163*, 327–337.

(17) Hartman, W. H.; Uzello, L.; Sobin, L. H.; Stalsberg, H. *Histological Typing of Breast Tumors*, 2nd ed.; World Health Organization (WHO): Geneva, Switzerland, 1981; pp 15−25.

(18) Negoescu, A.; Lorimier, P.; Labat-Moleur, F.; Drouet, C.; Robert, C.; Guillermet, C.; Brambilla, C.; Brambilla, E. *In situ* apoptotic cell labeling by the tunnel method: Improvement and evaluation on cell preparations. *J. Histochem. Cytochem.* **1996**, *44*, 959–968.

(19) Hamilton, J. G.; Comai, K. Rapid separation of neutral lipids, free fatty acids and polar lipids using prepacked silica sep-pak columns. *Lipids* **1999**, *23*, 1146–1149.

(20) Yasuda, M.; Nishizawa, T.; Ohigashi, H.; Tanaka, T.; Hou, D.; Colburn, N. H.; Murakami, A. Linoleic acid metabolite suppresses skin inflammation and tumor promotion in mice: Possible roles of programmed cell death 4 induction. *Carcinogenesis* **2009**, *30*, 1209–1216.

(21) Fischer, S. M.; Leyton, J.; Lee, M. L.; Locniskar, M.; Belury, M. A.; Maldve, R. E.; Slaga, T. J.; Bechtel, D. H. Differential effects of dietary linoleic acid on mouse skin-tumor promotion and mammary carcinogenesis. *Cancer Res.* **1992**, *52*, 2049s–2054s.

(22) Tisdale, M. J. Biology of cachexia. *J. Natl. Cancer Inst.* **1997**, *89*, 1763–1773.
(23) Monjazeb, A. M.; High, K. P.; Connoy, A.; Hart, L. S.; Koumenis, C.; Chilton, F. H. Arachidonic acid-induced gene expression in colon cancer cells. *Carcinogenesis* **2006**, *27*, 1950–1960.
(24) Majumder, B.; Wahle, K. W.; Moir, S.; Schofield, A.; Choe, S. N.; Farquharson, A.; Grant, I.; Heys, S. D. Conjugated linoleic acids (CLAs) regulate the expression of key apoptotic genes in human breast cancer cells. *FASEB J.* **2002**, *16*, 1447–1449.
(25) Kuwana, T.; Smith, J. J.; Muzio, M.; Dixit, V.; Newmeyer, D. D.; Kornbluth, S. Apoptosis induction by caspase-8 is amplified through the mitochondrial release of cytochrome *c*. *J. Biol. Chem.* **1998**, *273*, 16589–16594.
(26) Li, H.; Zhu, H.; Xu, C. J.; Yuan, J. Cleavage of Bid by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **1998**, *94*, 491–501.
(27) Liu, K. L.; Belury, M. A. Conjugated linoleic acid reduces arachidonic acid content and PGE_2 synthesis in murine keratinocytes. *Cancer Lett.* **1998**, *127*, 15–22.
(28) Papadimitriou, A.; King, A. J. F.; Jones, P. M.; Persaud, S. J. Anti-apoptotic effects of arachidonic acid and prostaglandin E2 in pancreatic β-cells. *Cell. Physiol. Biochem.* **2007**, *20*, 607–616.
(29) Adam-Klages, S.; Schwandner, R.; Lüschen, S.; Ussat, S.; Kreder, D.; Krönke, M. Caspase-mediated inhibition of human cytosolic phospholipase A2 during apoptosis. *J. Immunol.* **1998**, *161*, 5687–5694.
(30) Elstner, E.; Müller, C.; Koshizuka, K.; Williamson, E. A.; Park, D.; Asou, H.; Shintaku, P.; Said, J. W.; Heber, D.; Koeffler, H. P. Ligands for peroxisome proliferator-activated receptor γ and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells *in vitro* and in BNX mice. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 8806–8811.
(31) McCarty, M. F. Activation of PPARγ may mediate a portion of the anticancer activity of conjugated linoleic acid. *Med. Hypotheses* **2000**, *55*, 187–188.
(32) Schwab, M.; Reynders, V.; Loitsch, S.; Shastri, Y. M.; Steinhilber, D.; Schröder, O.; Stein, J. PPARγ is involved in mesalazine-mediated induction of apoptosis and inhibition of cell growth in colon cancer cells. *Carcinogenesis* **2008**, *29*, 1407–1414.
(33) Trosko, J. E.; Ruch, R. J. Cell−cell communication in carcinogenesis. *Front. Biosci.* **1998**, *3*, 208–236.
(34) Trosko, J. E.; Ruch, R. J. Gap junctions as targets for cancer chemoprevention and chemotherapy. *Curr. Drug Targets* **2002**, *3*, 465–482.

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