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Differential Inhibitory Effects of Conjugated Linoleic Acid Isomers on Mouse Forestomach Neoplasia Induced by Benzo(*a*)pyrene

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The differential anticarcinogenic activity of conjugated linoleic acid (CLA) isomers, including *c*9,*t*11-CLA, *t*10,*c*12-CLA, and *t*,*t*-CLA, was examined in a mouse forestomach carcinogenesis regimen induced by benzo(*a*)pyrene (BP). Female ICR mice (6−7 weeks of age, 26 ± 1 g) were divided into six groups (30 mice/group, 5 mice/cage): control, linoleic acid, CLA, *c*9,*t*11-CLA, *t*10,*c*12-CLA, and *t*,*t*-CLA. Each mouse was orally given 0.1 mL of sample and 0.1 mL of olive oil on Monday and Wednesday and BP (2 mg in 0.2 mL of olive oil) on Friday. This cycle was repeated four times. Twenty-three weeks later, the experiment was terminated for tumor analysis. *t*,*t*-CLA significantly reduced ($p < 0.05$) both tumor number and tumor size per mouse, relative to CLA and *c*9,*t*11-CLA, but similar to *t*10,*c*12-CLA. Reduction in tumor incidence by *t*,*t*-CLA (84.6%) was similar to that by CLA, c9,*t*11-CLA, and *t*10,*c*12-CLA, but it was significantly reduced ($p < 0.05$), relative to 100% linoleic acid and control. *t*,*t*-CLA elevated the apoptotic index to 35%, relative to 23% for CLA, 21% for *c*9,*t*11-CLA, 29% for *t*10,*c*12-CLA, 7% for linoleic acid, and 4% for control. *t*,*t*-CLA up-regulated the expression of the Bax gene and activated caspase-3 enzymes but down-regulated expression of the Bcl-2 gene. Cytosolic phospholipase A_2 activity was not affected by the CLA isomers tested. These results suggest that *t*,*t*-CLA has superior anticarcinogenic potential on BP-induced mouse forestomach neoplasia to CLA, *c*9,*t*11-CLA, and *t*10,*c*12-CLA, via the induction of apoptosis through mitochondrial dysfunction.

KEYWORDS: Conjugated linoleic acid (CLA); *t*,*t*-CLA; mouse forestomach neoplasia; benzo(*a*)pyrene; apoptosis

INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term for positional and geometric isomers of octadecadienoic acid (C18:2) with a conjugated double bond system. A CLA was first isolated from fried ground beef (*1*) and was subsequently found in a variety of dairy foods and ruminant-derived meats (*2*, *3*). CLA, which is synthesized from linoleic acid (LA) by alkaline isomerization, contains all possible CLA isomers: *t*10,*c*12-CLA and *c*9,*t*11-CLA are major isomers (*1*, *4*), and *t*,*t*-CLA, containing *t*7,*t*9-, *t*8,*t*10-, *t*9,*t*11-, *t*10,*t*12-, *t*11,*t*13-, and *t*12,*t*14-CLA isomers, are minor constituents (*5*). CLA has shown potent anticancer activity for carcinogen-induced carcinogenesis in several animals and human cancer cells (*6*), as well as exhibiting other biological activities (*7*, *8*). The anticarcinogenic potentials of CLA and its individual isomers, *t*10,*c*12-CLA and *c*9,*t*11-CLA, have been extensively studied (*6*, *9*).

Individual CLA isomers exhibit different anticancer activities. In human cancer cells, *t*10,*c*12-CLA isomer exhibits a higher potency on HT-29 colorectal cancer cells (*10*), MG-63 osteosarcoma cells (*11*), and MCF-7 breast cancer cells (*12*), relative to *c*9,*t*11-CLA. Meanwhile, diets containing 1% *c*9,*t*11-CLA and *t*10,*c*12-CLA are equally effective in reducing tumors and inducing apoptosis in the colonic mucosa of rats treated with 1,2-dimethylhydrazine (DMH) (*13*) and in decreasing turmorigenesis of *N*-methyl-*N*-nitrosourea (MNU)-induced rat mammary tumors (*14*). In contrast, only a few studies for *t*,*t*-CLA (*11*, *12*, *15*) or its individual *t*,*t*-CLA isomer, *t*9,*t*11-CLA (*16*−*18*), have been conducted in cancer cell lines and chemically induced animal carcinogenesis.

It is interesting that descriptions of *t*,*t*-CLA's anticarcinogenic action and that of its isomer have steadily appeared in the

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scientific literature and that this activity has been consistently shown to be more potent than other CLA isomers investigated to date. An osteosarcoma cell line, MG-63 (*11*), and a breast cancer cell line, MCF-7 (*12*), are more sensitive to *t*,*t*-CLA than *c*9,*t*11-CLA and *t*10,*c*12-CLA. Furthermore, *t*9,*t*11-CLA exhibits a strong antiproliferative activity on colon Caco-2, SW480, and HT-29 cancer cells (*16*, *17*) and a stronger anticancer activity in azoxymethane (AOM)-induced colonic aberrant crypt foci in rats (*18*).

Given these data, the anticarcinogenic activity of *t*,*t*-CLA must be evaluated on various cancer cell lines and chemical- or cancer cell-induced animal carcinogenesis models. Molecular mechanisms by which CLA and its isomers act as anticarcinogens are not well understood. However, many studies related to CLA isomers have demonstrated that apoptosis is one mechanistic action mediated by mitochondrial dysfunction (*6*, *11*, *12*). Hence, it is important to compare the anticarcinogenic potency of CLA isomers on chemically induced animal carcinogenesis through apoptosis mediated by mitochondrial dysfunction.

In the present study, the anticarcinogenic potency of CLA, *c*9,*t*11-CLA, *t*10,*c*12-CLA, and *t*,*t*-CLA was investigated on benzo(*a*)pyrene (BP)-induced mouse forestomach neoplasia, with reference to LA. The possible anticarcinogenic molecular mechanism was also elucidated.

MATERIALS AND METHODS

Materials. BP, Mayer's hematoxylin, RIPA buffer, Ac-DEVD-pNA, leupeptin, and pepstatin were obtained from Sigma-Aldrich (St. Louis, MO). LA (99.0%) was obtained from Nu Check PREP (Elysian, MN). The *i*-genomic CTB DNA Extraction Mini Kit was obtained from iNtRON Biotechnology (Seongnam, Republic of Korea). Rabbit polyclonal $cPLA_2$ antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal Bcl-2 and Bax antibodies and antirabbit and horseradish peroxidase (HRP)-conjugated mouse IgG were purchased from Delta Biolabs (Campbell, CA). Monoclonal caspase-8 antibody was obtained from Oncogene Research Products (San Diego, CA). ApopTaq peroxidase in situ apoptosis detection kit was obtained from Intergen (Burlington. MA). Vectastain ABC kits were purchased from Vector Laboratories (Burlingame, CA). The enhanced chemiluminescence (ECL) Western blotting kit was purchased from Amersham Biosciences (Buckinghamshire, United Kingdom). The Frag EL DNA fragmentation detection kit was purchased from Calbiochem (Burlingame, CA). All other reagents used were of analytical grade from Sigma-Aldrich.

Preparation of CLA Isomers. CLA was synthesized from LA by alkaline isomerization (*19*). The *c*9,*t*11- and *t*10,*c*12-CLA isomers were prepared from CLA methyl ester by low-temperature crystallization at −68 and −71 °C in conjunction with urea treatment (*19*). *t*,*t*-CLA was prepared from CLA using the BF_3/methanol previously described (*4*). The purity of CLA isomer samples was 97.1% for CLA, 89.5% for *c*9,*t*11-CLA, 99% for *t*10,*c*12-CLA, and 99% for *t*,*t*-CLA when analyzed by gas chromatography (*19*). *t*,*t*-CLA was composed of 0.7% *t*7,*t*9-CLA, 8.3% *t*8,*t*10-CLA, 41.0% *t*9,*t*11-CLA, 41.6% *t*10,*t*12-CLA, 7.8% *t*11, *t*13-CLA, and 0.6% *t*12,*t*14-CLA analyzed by silver high-performance liquid chromatography (*5*).

Animal Experiment. Female 6−7 week old ICR mice purchased from Hochang Science (Daegu, Republic of Korea) were housed in polycarbonate cages (five mice/cage) in a temperature (23 ± 2 °C)- and humidity (55 ± 3%)-controlled facility. Mice were permitted free access to water and chow diet (Hochang Science). One week later, the animals were randomized by body weight and divided into six groups (30 mice/group): control, LA, CLA, *c*9,*t*11-CLA, *t*10,*c*12-CLA, and *t*,*t*-CLA. They were subjected to a forestomach tumorigenesis regimen as described previously (*20*). On Monday and Wednesday, each animal was given (p.o.) 0.1 mL of sample (LA, CLA, or CLA isomer) mixed with 0.1 mL of olive oil. Control mice were given (p.o.) 0.1 mL of saline mixed with 0.1 mL of olive oil. On Friday, all animals were given 2 mg of BP in 0.2 mL of olive oil. This sequence was repeated for 4 weeks. Beginning with the first intubation and continuing thereafter, body weight and food intake were recorded once weekly. All surviving mice were sacrificed 23 weeks after the first dose of BP by the means of complete necropsies. Mice care and experimental procedures were in accordance with Gyeongsang National University Animal Ethics Guidelines (GNU-LA-17).

Tumor Analysis. Forestomach tissues were withdrawn and fixed in 10% phosphate-buffered formalin (pH 7.3). The forestomach was split longitudinally, and tumors 1 mm or larger were measured using a digimatic caliper (Mitutoyo, Kawasaki, Japan). Tumor numbers and incidence were recorded. The formalin-fixed forestomach tissues were paraffin-embedded, and 4 μm thick sections were cut and stained with hematoxylin and eosin (H&E), prior to histological examination (*21*).

Transferase-Mediated dUTP Nick End-Labeling (TUNEL) Assay. Terminal deoxynucleotidyl TUNEL was conducted according to the method described previously (*22*), using an ApopTaq peroxidase in situ apoptosis detection kits. Forestomach tissue specimens (4 μm thick) were observed using a model DM6000B microscope (Leica, Wetzlar, Germany). With the TUNEL method, five non-necrotic fields were randomly selected in each histological specimen, and in each field, the number of apoptotic positive nuclei was recorded as number per 100 cells.

DNA Fragmentation and Immunohistochemical Analysis. The forestomach tissues were frozen by the slow addition of liquid nitrogen and then disrupted and homogenized completely. DNA was extracted from the homogenate, using a *i*-genomic CTB DNA Extraction Mini Kit (*11*). For immunohistochemical analysis of Bcl-2 and Bax proteins, forestomach tissues were freshly removed and fixed in 10% phosphate-buffered formalin (pH 7.3). The level of Bcl-2 and Bax proteins was measured by the avidin−biotin−peroxidase complex method, using a Vectastain ABC kits (*23*).

Western Blotting. Three forestomachs obtained from each group were pooled and homogenized in RIPA buffer [150 mM NaCl, 0.1% NP40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), and 50 mM Tris, pH 7.4] containing protease inhibitors, 21 μM leupeptin, and 15 μM pepstatin. Cell lysate containing 40 μg of total protein, as determined by the Bradford protein assay method (*24*), was used for 10% SDS-polyacrylamide gel electrophoresis, and the resolved proteins were examined by Western blot as described previously (*25*). Bound antibodies were detected with the aid of an ECL Western blotting detection kit. The relative protein levels were determined using the software for the Kodak ID Scientific Imaging System.

Measurement of Caspase-3 Activity. The activity of caspase-3 was assayed based on the spectrophotometric detection of the chromatophore pNA after cleavage from the labeled substrate DEVD-pNA by caspase-3-like proteases (*26*). The DEVDase activity was evaluated by measuring proteolytic cleavage of chromogenic substrate Ac-DEVD-pNA used as the substrate for caspase-3. The absorbance of enzymatically released pNA was measured at 405 nm, using a Beckman DU 650 spectrophotometer.

Statistical Analysis. The results are presented as means ± SDs. Statistical analysis was carried out using analysis of variance (ANOVA) followed by Duncan's multiple range tests. Treatment mean differences with $p < 0.05$ were considered statistically significant.

RESULTS

Reduction of Forestomach Neoplasia. Table 1 shows the inhibition of BP-induced mouse forestomach neoplasia by CLA isomers. Control mice and LA-treated mice invariably showed a thickened and shrunken forestomach with a 100% tumor incidence. The tumor incidence in mice treated with CLA and CLA isomers was significantly ($p < 0.05$) different from control mice and LA-treated mice. The tumor incidence in *t*,*t*-CLA-treated mice (84.6%) was significantly different from that of control mice but was similar to CLA-treated mice (89.3%), *c*9,*t*11-CLA-treated mice (88.9%), and *t*10,*c*12-CLA-treated mice (89.3%). The *t*,*t*-CLA treatment significantly ($p < 0.05$) reduced tumor numbers as compared to control, LA, and *c*9,*t*11-CLA treatments but not *t*10,*c*12-CLA. Mice treated with *t*,*t*-CLA had 3.3 tumors/mouse, while mice treated with LA and control mice

Table 1. Reduction of BP-Induced Forestomach Neoplasms in Female ICR Mice by CLA Isomers

treatment	mouse survived[a] (%)	tumor incidence[b] (%)	mean size[c](mm)	tumor multiplicity	
				tumors /mouse	tumors/tumor- bearing mouse
control	27	100 a	2.5 ± 1.2[d]a	6.4 ± 1.5 a	6.4 ± 1.8 a
LA	26	100 a	2.5 ± 1.0 a	6.7 ± 1.1 a	6.7 ± 1.4 a
CLA	28	89.3 b	1.9 ± 0.6 b	5.1 ± 1.0 b	5.8 ± 1.2 a
*c*9,*t*11-CLA	27	88.9 b	1.9 ± 1.1 b	5.3 ± 0.9 b	6.0 ± 1.4 a
*t*10,*c*12-CLA	28	89.3 b	1.8 ± 1.2 b	4.8 ± 1.0 bc	5.4 ± 1.9 bc
t,*t*-CLA	26	84.6 b	1.7 ± 0.9 b	3.3 ± 0.5 c	4.5 ± 1.5 c

[a] Mice surviving from the initial 30 at termination time. [b] Percentage of tumor-bearing mice against mice surviving at termination time. Significance was tested by X^2. [c] Tumors >1 mm in size. [d] Mean ± SD. Means with different small superscript letters represent statistical significance at $p < 0.05$ by Duncan's multiple range test.

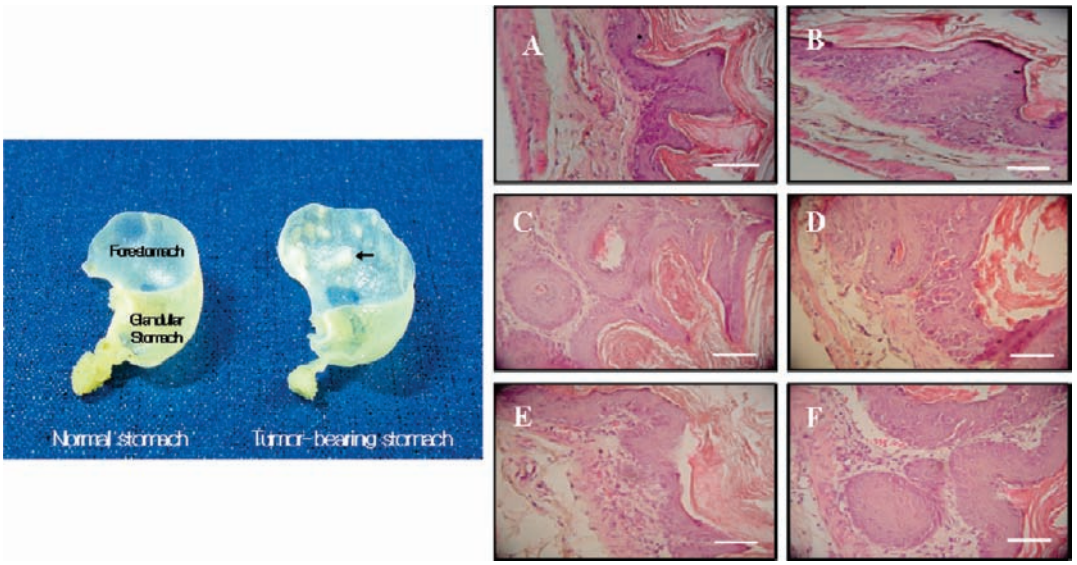

Figure 1. Typical features of forestomach (**left**) and histochemical analysis of forestomach tissues (**right**) of BP-treated female ICR mice by CLA isomers. Panel identification: control (**A**), LA (**B**), CLA (**C**), *c*9,*t*11-CLA (**D**), *t*10,*c*12-CLA (**E**), and *t*,*t*-CLA (**F**) treatments. The bars represent 50 μm (×400) in each panel.

contained 6.7 and 6.4 tumors/mouse, respectively. Mice treated with CLA, *c*9,*t*11-CLA, and *t*10,*c*12-CLA developed 5.1, 5.3, and 4.8 tumors/mouse, respectively. A similar effect of *t*,*t*-CLA was seen in tumors/tumor-bearing mice. The average size of the forestomach neoplasm in *t*,*t*-CLA-treated mice was smaller than in control mice and other CLA-treated mice. Forestomach neoplasm > 5 mm was not found in the forestomach treated with *t*,*t*-CLA but was evident in the forestomach of mice treated with CLA, *c*9,*t*11-CLA, and *t*10,*c*12-CLA. These results indicate that *t*,*t*-CLA exhibited the most potent anticarcinogenic activity on BP-induced mouse forestomach tumorigenesis, but *c*9,*t*11-CLA and *t*10,*c*12-CLA isomers exhibited an equal anticarcinogenic activity.

Over a period of the experiment, the average body weights of mice treated with CLA and CLA isomers were slightly reduced but not significantly different from those of mice treated with control or LA (data not shown). There were no differences in food intake among treatment groups during the experimental period (data not shown).

Histological Examination. Microscopic examination revealed neoplasia at the squamous mucosa of the forestomach of mice exposed to BP (**Figure 1**). Neoplasms of the forestomach consisted of hyperplastic mucosal epithelium and fibers keratinized from the epithelial cells. In BP-treated control mice, the forestomach neoplasm was of mild severity with an increased thickness to 9−12 cell layers or greater as compared to the vehicle control (data not shown). The superficial layers of keratin fibers on the mucosal surface also were slightly increased in thickness, relative to vehicle control (data not shown). No significant neoplasia was observed in the forestomach of mice treated with CLA and CLA isomers. The thickness of forestomach walls was increased by epithelial hyperplasia. In addition, examination of H&E-stained glandular stomach sections from vehicle control and BP-treated groups revealed no morphological changes (data not shown).

Induction of Apoptosis. Apoptosis may be responsible for the inhibition of BP-induced forestomach neoplasia by CLA isomers, as shown in **Table 1**. To test this hypothesis, apoptosis was determined by TUNEL and DNA fragmentation assays for the forestomach tumors from mice treated with CLA isomers (**Figure 2**). TUNEL analysis revealed a frequent occurrence of darkly stained TUNEL-positive nuclei in the forestomach epithelium of mice treated with CLA and CLA isomers, as compared with control and LA-treated forestomach, which had only occasional incidences of apoptotic cells. Mice treated with *t*,*t*-CLA had a higher increase in apoptosis than mice treated with the CLA and other CLA isomers, based on the percentage of apoptotic cell index: 4.5 (control), 7.1 (LA), 22.6 (CLA), 21.6 (*c*9,*t*11-CLA), 29.8 (*t*10,*c*12-CLA), and 35.5 (*t*,*t*-CLA). *t*,*t*-CLA reduced ($p < 0.05$) the index value as compared to that of CLA, *c*9,*t*11-CLA, and *t*10,*c*12-CLA. *t*,*t*-CLA produced a distinct smearing of DNA fragments, a typical characteristic of cells undergoing apoptosis (*27*), in forestomach treated with BP. *c*9,*t*11-CLA and *t*10,*c*12-CLA isomers increased the quantity of this smearing DNA, but the quantity was evident in *t*,*t*-CLA-treated tumors. These suggest that *t*,*t*-CLA increased apoptosis as compared with *c*9,*t*11-CLA and *t*10,*c*12-CLA isomers.

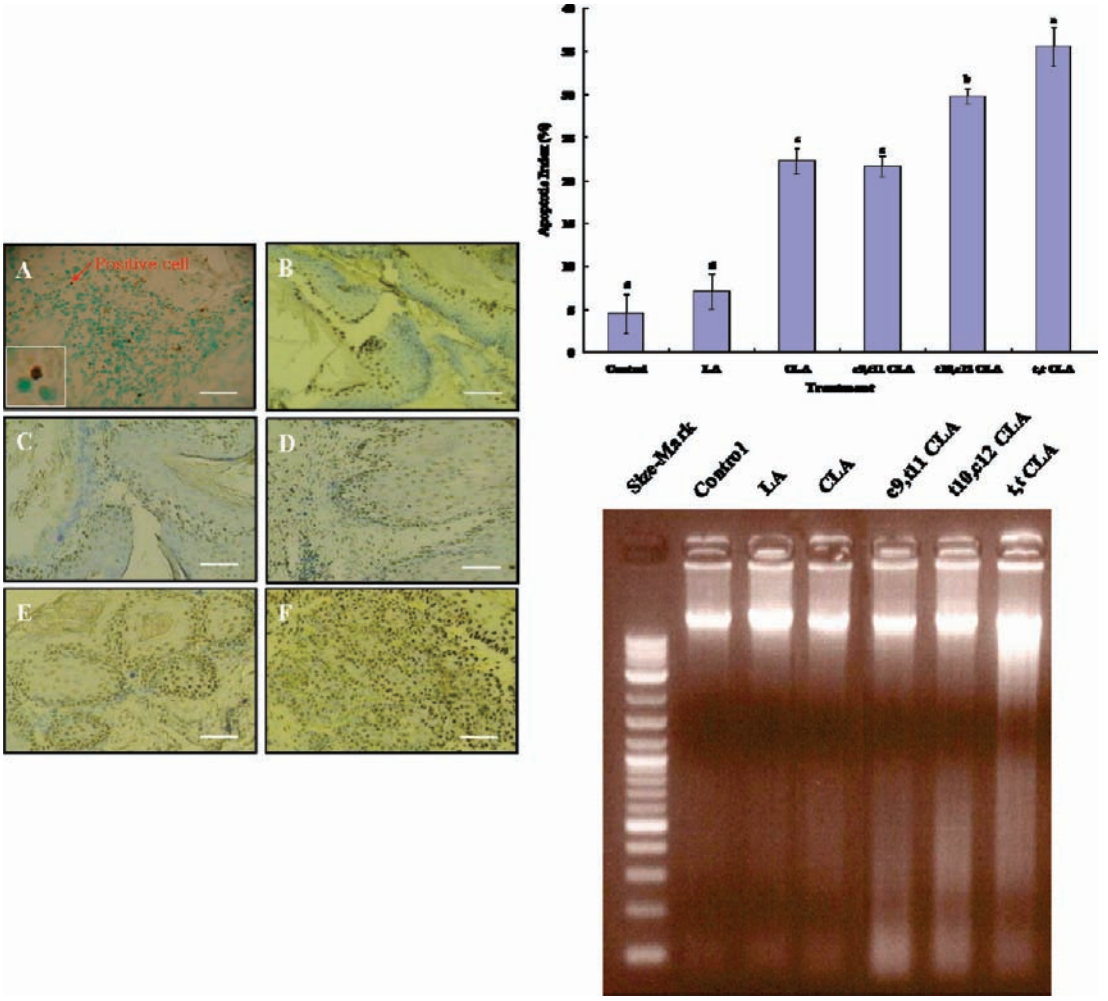

Figure 2. Induction of apoptosis in BP-treated forestomach tissues from female ICR mice by CLA isomers. Panel identification: control (**A**), LA (**B**), CLA (**C**), *c*9,*t*11-CLA (**D**), *t*10,*c*12-CLA (**E**), and *t*,*t*-CLA (**F**) treatments. The bars represent 50 μm ($\times$400). Data in the apoptotic index are represented as means $\pm$ SDs ($n = 3$). Means with different small letters represent statistical significance at $p < 0.05$ by Duncan's multiple range test.

Expression of Apoptosis-Related Genes. The process of apoptosis is dependent on the type of apoptosis regulatory gene products that may be either death antagonist (e.g., Bcl-2 and Bcl-xL) or death agonist (e.g., Bax and Bad, p53); hence, the expression of Bcl-2 and Bax genes was determined to establish the relation of apoptosis in BP-induced forestomach neoplasia by dye-staining and Western blotting assays (**Figure 3**). *t*,*t*-CLA-treated forestomach epithelia revealed a stronger staining of Bax protein than CLA-, *c*9,*t*11-CLA-, and *t*10,*c*12-CLA-treated forestomach epithelia. In accordance with progressive apoptosis, *t*,*t*-CLA significantly ($p < 0.05$) increased the percentages of Bax positive cells than CLA, *c*9,*t*11-CLA, and *t*10,*c*12-CLA, and *t*10,*c*12-CLA significantly ($p < 0.05$) increased than CLA and *c*9,*t*11-CLA. These results were further confirmed by Western blotting analysis of Bax protein as shown in **Figure 3**. The *t*,*t*-CLA-treated forestomach neoplasms induced a higher level of expression of Bax as compared to CLA, *c*9,*t*11-CLA, and *t*10,*c*12-CLA treatments. Bax expression by *t*10,*c*12-CLA was slightly higher than that by CLA and *c*9, *t*11-CLA.

In contrast, the expression level of Bcl-2 reduced in *t*,*t*-CLA-treated forestomach neoplasm, relative to CLA, *c*9,*t*11-CLA, and *t*10,*c*12-CLA (**Figure 4**). The immunohistochemical staining of Bcl-2 expression was typically strong in the basal and proliferative areas of BP-treated forestomach but weak and infrequent in *t*,*t*-CLA-treated epithelium. The percentage of Bcl-2 positive cells in forestomach tissues was significantly ($p < 0.05$) reduced by *t*,*t*-CLA treatment, relative to CLA and *c*9,*t*11-CLA treatments, but not to *t*10,*c*12-CLA treatment. Meanwhile, Bcl-2 expression in forestomach neoplasm tissues was also significantly ($p < 0.05$) reduced by CLA, *c*9,*t*11-CLA, and *t*10,*c*12-CLA treatments as compared to control- or LA-treated mice. As a result, the expression of Bcl-2 protein was down-regulated in forestomach neoplasm induced by BP. *t*,*t*-CLA and *t*10,*c*12-CLA effects on the reduction of Bcl-2 expression in forestomach neoplasm were apparent in Western blotting analysis (**Figure 4**). Overall, the results implicated the stronger anticarcinogenic activity of *t*,*t*-CLA in BP-induced forestomach neoplasia with a reciprocal regulation of Bax and Bcl-2 gene expression.

Caspases play key roles in apoptosis (*28*). To determine whether caspase activation is a required signal event for apoptosis and whether increases in caspase activity coincided with apoptotic cell death, caspase-3 activity was measured in forestomach tissues treated with CLA isomers. As shown in **Figure 5**, CLA isomer treatments significantly ($p < 0.05$) elevated caspase-3 activity, relative to control and LA treatments, and *t*,*t*-CLA and *t*10,*c*12-CLA have a stronger efficacy by approximately 2.0- and 1.8-fold, respectively, to control treatment. Because both TNF and Fas-mediated apoptotic signals commonly activate caspase-8 (*29*), it became of interest to investigate whether *t*,*t*-CLA treatment activated caspase-8 in forestomach noeplasia induced by BP.

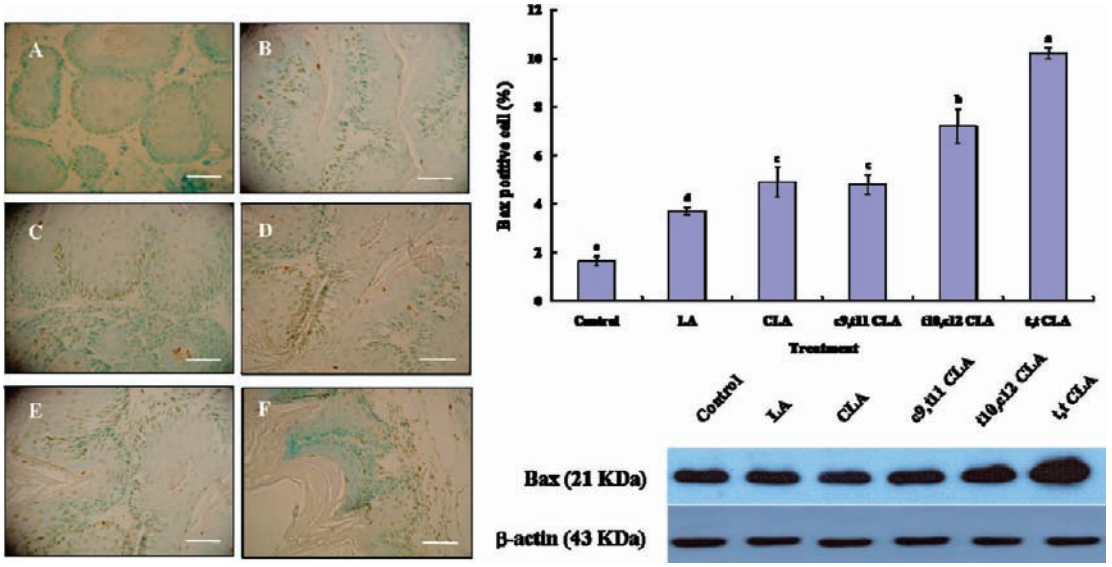

Figure 3. Up-regulation of Bax protein in BP-treated female ICR mice forestomach tissues by CLA isomers. Panel identification: control (**A**), LA (**B**), CLA (**C**), *c*9,*t*11-CLA (**D**), *t*10,*c*12-CLA (**E**), and *t*,*t*-CLA (**F**) treatments. The bars represent 50 μm ($\times$400). Data in Bax positive cells are represented as means $\pm$ SDs (n = 3). Means with different small letters represent statistical significance at $p < 0.05$ by Duncan's multiple range test.

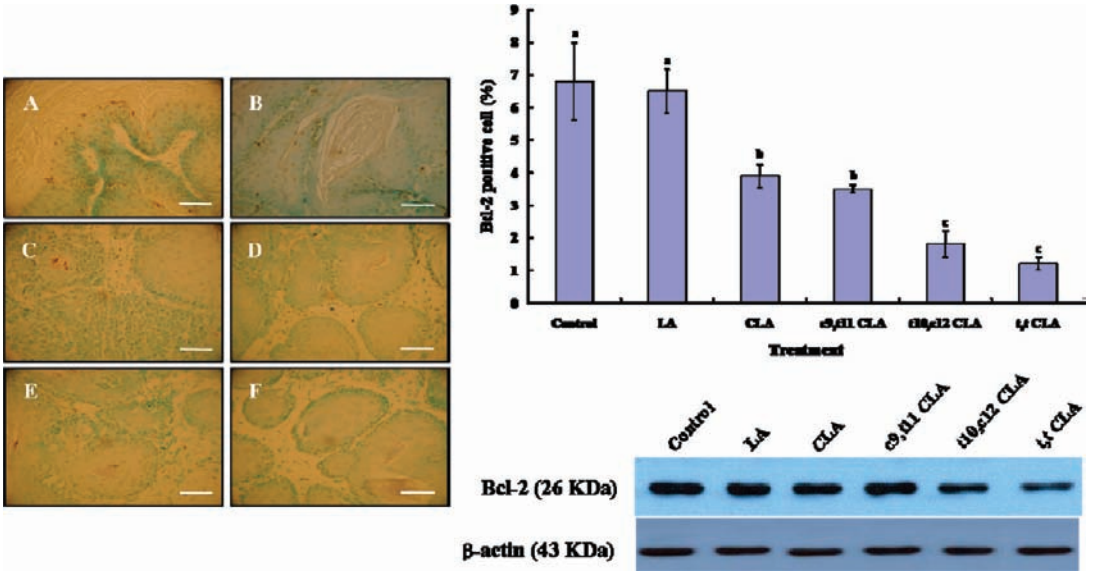

Figure 4. Down-regulation of Bcl-2 protein gene in BP-treated female ICR mice forestomach tissue by CLA isomers. Panel identification: localization of Bcl-2 protein in cells of tissues from control (**A**), LA (**B**), CLA (**C**), *c*9,*t*11-CLA (**D**), *t*10,*c*12-CLA (**E**), and *t*,*t*-CLA (**F**). The bars represent 50 μm ($\times$400). Data in Bcl-2 positive cells are represented as means $\pm$ SDs (n = 3). Means with different small letters represent statistical significance at $p < 0.05$ by Duncan's multiple range test.

The treatments of CLA, *c*9,*t*11-CLA, *t*10,*c*12-CLA, and *t*,*t*-CLA induced a significantly higher expression of caspase-8 ($p < 0.05$), resulting in an increase in the caspase-8 expression by 1.8-fold, relative to control, without significance between CLA isomers.

Tumors usually produce more prostaglandins (PGs) through arachidonic acid (AA) metabolism than their associated normal mucosa/submucosa (*30*), but the mechanisms are not fully understood. The key enzymes are cytosolic phospholipase A_2 ($cPLA_2$) and cyclooxygenase-2 (COX-2), which exist in an inducible form. In BP-induced forestomach neoplasia, $cPLA_2$ expression in forestomach tumors treated with *t*,*t*-CLA and *t*10,*c*12-CLA was similar to that in forestomach tumor tissues treated with LA and CLA. Hence, the inhibitory effect of *t*,*t*-CLA shown in **Table 1** was not related to the AA metabolism but rather was related to the apoptosis regulated by a reciprocal expression of Bax and Bcl-2 and activation of caspases-3.

DISCUSSION

CLA was previously shown to be an effective anticarcinogen against mouse forestomach carcinogenesis induced by BP (*20*). *c*9,*t*11-CLA and *t*10,*c*12-CLA have been intensively studied concerning their anticarcinogenic activities against chemically induced animals and human cancer cell lines (*6*, *9*), whereas the *t*,*t*-CLA isomer was long exempt from scientific concern, due to its minor constituents in CLA (*4*, *5*). However, interest in *t*,*t*-CLA has been steadily growing since we first reported that the anticarcinogenic potential of *t*,*t*-CLA in stomach cancer cell line, N87, is superior to *c*9,*t*11-CLA and *t*10,*c*12-CLA (*15*) in the MG-63 osteosarcoma cell line (*11*) and MCF-7 breast cancer cell line (*12*). Recently, *t*9,*t*11-CLA was shown to exhibit a strong antiproliferative activity on Caco-2, SW480, and HT-29 colon cancer cells (*16*, *17*) and also reduced the AOM-induced colonic aberrant crypt foci in rats (*18*).

However, the mechanistic action of tumor reduction by *t*,*t*-CLA or by its individual *t*,*t*-CLA isomers is poorly understood.

This study has clearly shown that the anticarcinogenic potential of *t*,*t*-CLA on BP-induced mouse forestomach turmorigenesis is superior to CLA, *c*9,*t*11-CLA, and *t*10,*c*12-CLA, through induction of apoptosis mediated by mitochondrial dysfunction. The anticarcinogenic potential of *t*,*t*-CLA, relative to *c*9,*t*11-CLA and *t*10,*c*12-CLA, was evaluated by tumor incidence and multiplicity, apoptosis and apoptosis-related gene expression, and enzyme activation. Our findings are consistent with the anticarcinogenic potential and apoptotic mechanism of *t*,*t*-CLA in human cancer cell studies including MG-63 (*11*), MCF-7 (*12*), and the colon cancer cells (*16*, *17*). Apoptosis through mitochondrial dysfunction can be induced in chemically induced animal studies by CLA isomers (*6*, *11*, *12*). The superior potency of anticarcinogenic activities of *t*,*t*-CLA, relative to *c*9,*t*11-CLA and *t*10,*c*12-CLA was directly related to the configuration at the double bonds of CLA isomers; *t*,*t*-CLA is a straight chain that resembles stearic acid (C18:0) and shows the strongest anticarcinogenic activity, whereas *c*9,*t*11-CLA resembles LA and shows the least activity (*11*, *12*). The present results were evaluated by measuring tumor multiplicity and some of biomarkers, such as Bax, Bcl-2, and caspase-3, at the end of experiment (23 weeks postinitiation of tumor with BP); hence, further studies could be performed to confirm these results by measuring biomakers at time-dependent events in apoptotic pathways. The present result is the first demonstration of potent apoptotic induction in BP-induced forestomach neoplasia by *t*,*t*-CLA and, as such, is important fundamental data that will pave the way for study of its mechanistic actions of biological activities. Because of the difficulties in the preparation of individual *t*,*t*-CLA isomers for animal studies, we did not examine the anticarcinogenic activity of individual *t*,*t*-CLA isomers in the present study, but further research should be performed to clarify anticarcinogenic efficacy of each *t*,*t*-CLA isomer.

For the induction of apoptosis in tumor cells, the expression of pro-apoptotic regulatory genes (Bax and Bcl-xS) was up-regulated, whereas the expression of antiapoptotic regulatory genes (Bcl-2 and Bcl-xL) must be down-regulated to induce apoptosis of tumor cells (*31*). We observed an increased level of Bax protein and a decreased level of Bcl-2 protein in BP-induced mouse forestomach neoplasia by CLA isomers (**Figures 3** and **4**), indicating the involvement of Bax and Bcl-2 genes in a reciprocally regulated fashion. The efficacy of *t*,*t*-CLA on the regulation of Bax gene was greater than that of *c*9,*t*11-CLA and *t*10,*c*12-CLA, but similar to regulation of Bcl-2 gene of *t*10,*c*12-CLA. It is well-known that Bax helps the release of cytochrome *c* from the mitochondrial matrix to cytosol and cytochrome *c* release activates caspase-3, which, in turn, executes apoptosis, as a primary mechanism of apoptosis (*32*). The Bax protein elevated by CLA isomers stimulates the release of cytochrome *c* from mitochondrial matrix to cytosol of MG-63 and MCF-7 cell lines. Consequently, cytochrome *c* in cytosol activated caspase-3 from procaspase-3 enzyme to induce apoptosis. Hence, Bax protein elevated by *t*,*t*-CLA, relative to *c*9,*t*11-CLA, and *t*10,*c*12-CLA in forestomach tumor cells treated with BP enhances the release of cytochrome *c* into cytosol, which, in turn, activates caspase-3 from pro-caspase-3 enzyme to induce the apoptosis of mouse forestomach tumor cells, with *t*,*t*-CLA being the most effective (**Figure 5**). This apoptosis induced by CLA isomers is in agreement with the antiproliferative activity of *c*9,*t*11-CLA and *t*10, *c*12-CLA exerted in human tumor cells by the apoptotic pathway via activation of caspase-3 (*11*, *12*, *33*). The mechanistic action of *t*,*t*-CLA is completely unknown, but its action could be similar to that of *c*9,*t*11-CLA and *t*10,*c*12-CLA.

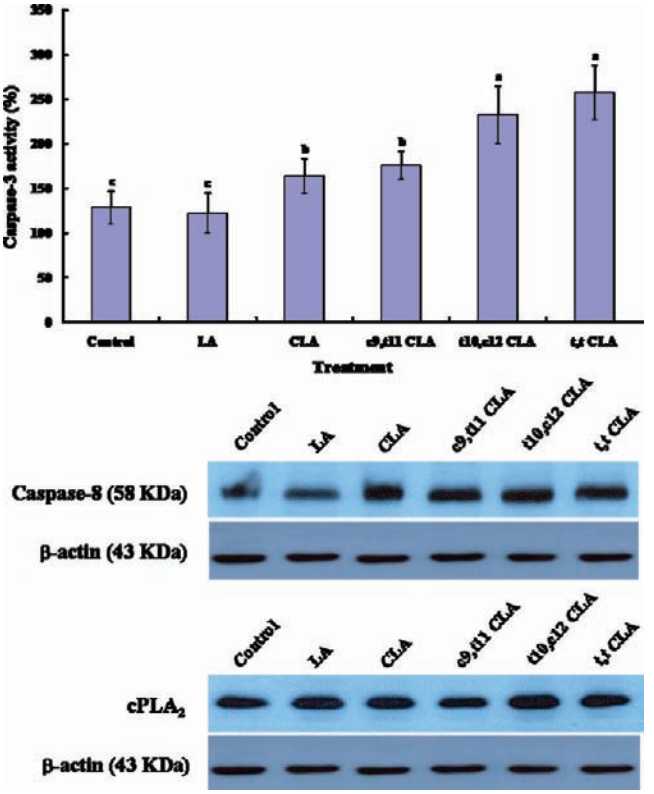

Figure 5. Differential effects of CLA isomers on the activation of caspase-3 and caspase-8 and expression of cPLA2 gene in BP-treated female ICR mice forestomach tissues. Data in caspase-3 activity are represented as means ± SDs (n = 3). Means with different small letters represent statistical significance at $p < 0.05$ by Duncan's multiple range test.

In addition to the caspase-3 pathway, CLA isomers activate capase-8 in BP-induced mouse forestomach tumor cells (**Figure 5**). Caspase-8 cleaves the Bcl-2 family member Bid and, in turn, induces cytochrome *c* release from mitochondria to active caspase-9 (*29*). The activated caspase-9 is then able to activate caspase-3, leading to DNA degradation, a hallmark event in apoptosis (*27*). Hence, we believe that all of the presently tested CLA isomers are involved in the activation of caspase-8 but that the efficacy of *t*,*t*-CLA is equal to CLA, *c*9,*t*11-CLA, and *t*10,*c*12-CLA isomers. These results imply that CLA and CLA isomers might be attributed to activation of the extrinsic apoptotic pathway.

Apoptosis is, in part, attributable to the alterations in fatty acid composition of membrane PLs, membrane fluidity, and eicosanoid metabolism (*32*, *34*). Release of AA in membrane PL is catalyzed by $cPLA_2$ and, in turn, oxides to PG by COX-2, which is commonly up-regulated in various forms of cancer to regulate apoptosis. No significant changes in $cPLA_2$ protein were observed from tumor tissues by CLA, *c*9,*t*11-CLA, *t*10,*c*12-CLA, and *t*,*t*-CLA (**Figure 5**). These might reflect the fact that the level of $cPLA_2$ proteins was measured in forestomach tissues at 23 weeks postintubation of CLA isomers. This might imply that at an early stage of tumorigenesis of BP-induced mouse forestomach, $cPLA_2$ is involved in the induction of apoptosis of tumor cells but not at a later stage.

In conclusion, the anticarcinogenic potency of *t*,*t*-CLA in BP-induced mouse forestomach turmorigenesis is greater than that of *c*9,*t*11-CLA but similar to *t*10,*c*12-CLA isomers. The anticarcinogenic action of these CLA isomers is attributed to the induction of apoptosis mediated by mitochondrial dysfunction, up-regulation of Bax gene expression caspase-3 enzyme activity, and down-regulation of Bcl-2 gene expression. The precise signaling event

involved in CLA-induced apoptosis in forestomach remains to be determined.

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