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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

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The growth inhibitory effect of a mixture of *trans,trans* conjugated linoleic acid isomers (*t,t* CLA) was investigated in a human breast cancer cell line, MCF-7, with references to *c*9,*t*11 CLA, *t*10,*c*12 CLA, and linoleic acid. The *t,t* CLA treatment effectively induced a cytotoxic effect in a time-dependent (0−6 days) and concentration-dependent (0−40 μM) manner, as compared to the reference and control treatments. The apoptotic parameters were measured on cells treated with 40 μM *t,t* CLA for 4 days. The occurrence of the characteristic morphological changes and DNA fragmentation confirmed apoptosis. The *t,t* CLA treatment led to an increase in the level of p53 tumor suppressor protein and Bax protein, but suppressed the expression of Bcl-2 protein. In addition, cytochrome *c* was released from the mitochondria into the cytosol, and the activation of caspase-3 led to the cleavage of poly(ADP-ribose) polymerase (PARP). Moreover, the composition of the linoleic and arachidonic acids was decreased in cellular membranes. These findings suggest that incorporation of *t,t* CLA in the membrane induces a mitochondria-mediated apoptosis that can enhance the antiproliferative effect of *t,t* CLA in MCF-7 cells.

KEYWORDS: Conjugated linoleic acid (CLA) isomers; *t,t* CLA isomer; apoptosis; human breast cancer cell line, MCF-7; Bax; Bcl-2

INTRODUCTION

Conjugated linoleic acid (CLA) refers to a group of polyunsaturated fatty acids that exist as positional (7,9; 8,10; 9,11; 10,12; 11,13; 12,14) and geometric (*cis*, *trans*) isomers of octadecadienoic acid (18:2). Since being discovered in grilled ground beef (*1*), the *c*9,*t*11 CLA isomer has been found to be a major form of naturally occurring CLA found in dairy products and ruminant meats (*2*). The *c*9,*t*11 CLA and *t*10,*c*12 CLA isomers are being studied both as a mixture and separately for their potential benefits in controlling obesity, improving immune function and insulin sensitivity, and altering lipid metabolism (*3*).

CLA isomers have been shown to act as anticancer agents in a number of rodent and human tumor model systems, including carcinogen-induced and transplanted mammary tumor models (*1, 4, 5*). Several recent reviews assessed the effects and mechanisms of CLA isomers in biological systems, including cancer (*5*). Although a number of biochemical and molecular mechanisms have been suggested to explain their anticarcinogenic effects, the apoptosis is the most important molecular mechanism in cancer cells (*6*). Meanwhile, a few studies reported that CLA isomers regulated the growth of human cancer cells and transplantable mammary tumor models (*7, 8*).

Synthetic CLA, which can be chemically synthesized from linoleic acid, typically consists of *c*9,*t*11 (40.8−41.1%), *t*10,*c*12 (43.5−44.9%), *t*9,*t*11, and *t*10,*t*12 (4.6−10%) isomers (*9*). New evidence indicates that the various biological activities of CLA result from the separate actions of these individual isomers acting synergistically (*10*). Kim et al. (*11*) showed that *t*10,*c*12 CLA reduced 5-hydroxyeicosatetraenoic acid production in human breast tumor cells. Ou et al. (*12*) also demonstrated that *t*10,*c*12 CLA triggers apoptosis of p53 mutant murine mammary tumor cells through the mitochondrial pathway, due to the targeting of Bcl-2. In addition, different cytotoxic mechanisms of *c*9,*t*11 CLA and *t*10,*c*12 CLA isomers were evaluated in several human cancer cell lines, gastric adenocarcinoma; col-

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orectal cancer; prostate cancer; and osteosarcoma cells, and showed that the anticancer effects of the individual CLA isomers are not equivalent (*10, 13–16*). A higher anticarcinogenic effect of *t*10,*c*12 CLA than of *c*9,*t*11 CLA has been also found in prostate cancer, colon cancer, and osteosarcoma cells and in the Min mouse model (*10, 13, 16*). Not much attention has been paid to the antiproliferative affects of the mixture of *t*,*t* conjugated linoleic acid isomers with double bonds at C7,C9; C8,C10; C9,C11; C10,C12; C11,C13; and C12,C14 (designated *t*,*t* CLA) on cancer cells. We recently found that *t*,*t* CLA inhibited the growth of an osteosarcoma cell line, MG-63, in a dose-dependent manner (*16*). Meanwhile, no reports are available on the growth inhibitory effect of *t*,*t* CLA in breast cancer cells.

The purpose of this study is to determine whether *t*,*t* CLA could inhibit the cell growth of MCF-7 human breast cancer cells with reference to *c*9,*t*11 CLA, *t*10,*c*12 CLA, and linoleic acids through the pathway involving Bax- and Bcl-2-mediated mitochondrial dysfunctions.

MATERIALS AND METHODS

Reagents. Hoechst 33258, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin (BSA), sodium selenite, L-ascorbic acid, α-tocopherol phosphate, phenylmethanesulfonyl fluoride (PMSF), leupeptin, and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM)/F-12, fetal bovine serum (FBS), 0.5% trypsin−5.3 mM ethylenediaminetetraacetic acid (EDTA), penicillin−streptomycin, phosphate-buffered saline (PBS), and transferrin were obtained from Gibco BRL (Rockville, MD). Rabbit polyclonal antibodies for Bcl-2, Bax, p53, and caspase-3 and anti-rabbit and anti-mouse IgG−horseradish peroxidase were purchased from Delta Biolabs (Vandell Way Campbell, CA). Rabbit polyclonal antibodies for cytochrome *c* and poly(ADP-ribose) polymerase (PARP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-β-actin was purchased from Sigma Chemical Co. (St. Louis, MO). The Wizard Genomic DNA purification kit was purchased from Promega (Madison, WI). Heptadecanoic acid (C17:0) and fatty acid standards were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents used were of analytical grade.

Preparations of CLA Isomers and Fatty Acid−Albumin Complexes. Synthetic CLA was prepared from linoleic acid by alkaline isomerization (*9*). The *c*9,*t*11 and *t*10,*c*12 CLA isomers were prepared from synthetic CLA methyl ester by low-temperature crystallization at −68 and −71 °C in conjunction with urea treatment (*9*). The *t*,*t* CLA was prepared from synthetic CLA, using the method described by Kim et al. (*17*). The purity of CLA isomer samples was found to be 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 (GC) as described below. The composition of the *t*,*t* CLA sample was 1.2% *t*7,*t*9 CLA, 4.1% *t*8,*t*10 CLA, 43.5% *t*9,*t*11 CLA, 42.4% *t*10,*t*12 CLA, 5.0% *t*11,*t*13 CLA, and 3.8% *t*12,*t*14 CLA when analyzed by Ag-HPLC (*17*). Each CLA isomer or linoleic acid was complexed with fatty acid-free BSA according to the method described by Van Greevenbroek et al. (*18*).

Cell Culture and Sample Treatment. Breast cancer MCF-7 cells (Korean Cell Line Bank, Seoul, Korea) were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in DMEM/F-12 medium supplemented with 10% FBS and penicillin−streptomycin (100 units/mL and 100 μg/mL, respectively) according to the method described by Kim et al. (*16*). In brief, cells that had been grown to 80% confluence were treated with 0.1% trypsin−1.06 mM EDTA to disperse the cells. The cells were seeded into either a 12-well culture plate at a density of 1×10^4 cells per well for cytotoxicity test or a culture dish (90 × 15 mm) at a density of 1×10^6 for the other experiments. After 24 h of incubation, the cells were starved of the serum by incubation for an additional 24 h in a DMEM/F-12 medium supplemented with 5 μg/mL of transferrin, 5 ng/mL of selenium, and 0.1 mg/mL of BSA.

For the cytotoxicity assay, two experiments were conducted. In the first experiment, the cells that had been starved of serum were incubated with *t*,*t* CLA (0−60 μM) in DMEM/F-12 medium, supplemented with ascorbic acid (50 ng/mL) and α-tocopherol phosphate (20 ng/mL), for a period of 6 days to determine an optimal *t*,*t* CLA concentration and incubation time. In the second experiment, the cytotoxicity of *t*,*t* CLA was compared to that of *c*9,*t*11 CLA, *t*10,*c*12 CLA, and linoleic acid under the optimal conditions determined; cells were incubated with 40 μM each of *t*,*t* CLA, *c*9,*t*11 CLA, *t*10,*c*12 CLA, and linoleic acid for 4 days. For the other experiments, the cells were incubated with 40 μM *t*,*t* CLA, 40 μM *c*9,*t*11 CLA, 40 μM *t*10,*c*12 CLA, and 40 μM linoleic acid for 4 days. Medium was replaced with fresh medium every 2 days. The exponentially growing cells were used throughout the experiments. The cells that had been trypsinized with 0.1% trypsin−1.06 mM EDTA were collected by centrifugation (1000 rpm, 10 min, 4 °C), resuspended in PBS, and stored at −20 °C until use.

Cell Viability Test. The viable cell number was estimated using the MTT assay (*19*). The absorbance was measured at 570 nm using an Anthos 2020 model microplate reader (Anthos Labtech Instruments, Wals., Austria). Data were represented as a growth rate, which is the ratio of viable cell numbers at a given day of incubation to the cell numbers at day 0.

DNA Fragmentation Assay. DNA was isolated from cells using a Wizard Genomic DNA purification kit from Promega. The DNA fragmentation pattern was analyzed by electrophoresis in a 1.2% agarose gel and was then visualized under UV light with ethidium bromide according to the method described by Allen et al. (*20*).

Hoechst 33258 Staining. The cells were fixed with 4% paraformaldehyde in PBS for 10 min. The cells were then permeabilized in PBS containing 0.1% Triton X-100 for 10 min, after which the nuclei were stained with Hoechst dye (10 μg/mL) for 10 min as described by Olkku et al. (*21*). Finally, the cells were observed with a fluorescence microscope (Carl Zeiss, Germany).

Western Blot Analysis. The cells were lysed in buffer (50 mM Tris-HCl, pH 7.4 containing 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, and 10% glycerol) that contained the protease inhibitors leupeptin (10 μg/mL) and PMSF (2 mM) as described by Kim et al. (*16*). The protein concentration was determined according to the method described in the Bio-Rad Protein Assay manual. Western blotting was performed as described by Ochoa et al. (*13*). In addition, bound antibodies were detected with the aid of an enhanced chemiluminescence (ECL) Western blotting detection kit (Amersham Biosciences, Buckingham, U.K.). The relative protein levels were determined using the software for the Kodak ID Scientific Imaging System.

Mitochondrial Cytochrome *c* Release Assay. Mitochondrial and cytosolic fractions from the cells were prepared using a previously reported method (*16*). In brief, the cells were harvested and washed with PBS and were then resuspended at 5×10^7 cells/mL in an extraction buffer (20 mM HEPES, pH 7.5, containing 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 PMSF, and 250 mM sucrose). The cells were then homogenized using a tight-fitting Dounce homogenizer (Seoul, Korea). The homogenates were centrifuged at 750*g* for 10 min at 4 °C. The supernatants were centrifuged at 10000*g* for 15 min (4 °C) using a Beckman ultracentrifuge (S.A., Gagny, France), and the pellets were resuspended in 0.5 mL of extraction buffer (designated the mitochondrial fraction). The supernatants that had been separated by centrifugation at 10000*g* were further centrifuged at 100000*g* for 1 h (4 °C), and the resulting supernatants were collected (designated the cytosol fraction). The cytochrome *c* of both the cytosolic and mitochondrial fractions was analyzed by probing with an anti-cytochrome antibody using the standard Western blot analysis protocol.

Preparation and Analysis of Membrane Fatty Acids. *Preparation of Membrane Lipids.* The isolation of the plasma membrane of the cells and the analysis of fatty acid composition in the plasma membrane were conducted according to the method described by Kim et al. (*16*). In brief, cells were homogenized in 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 200 mM sucrose, and 1 mM PMSF with a tight-fitting Dounce homogenizer (Seoul, Korea). After removal of the nuclei and cell debris from the homogenate by centrifugation at 7500*g* for 5 min at 4 °C, the supernatant was centrifuged at 45000*g* for 40

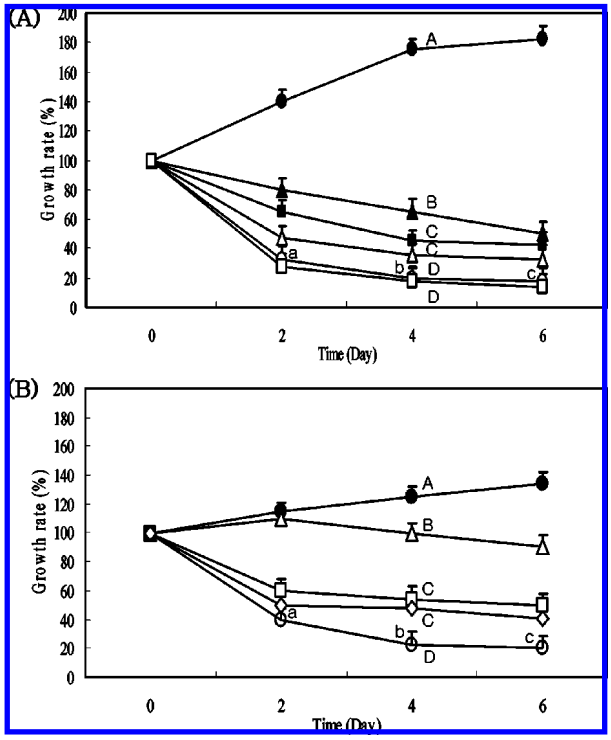

Figure 1. Growth rate of MCF-7 cells treated with *t,t* CLA for 6 days: (**A**) cells treated with various concentrations of *t,t* CLA (5 μM, ▲; 10 μM, ■; 20 μM, △; 40 μM, ○; 60 μM, □) and control cells (●); (**B**) cells treated with 40 μM *t,t* CLA (○), *c*9,*t*11 CLA (□), *t*10,*c*12 CLA (◇), and linoleic acid (△) and control cells (●). Values of the percent cell viability against the control at day 0 are indicated as means ± SD ($n = 3$). Means with different lower case letters on different concentration lines or with different upper case letters on different concentration lines at the same incubation time denote a significant difference ($p < 0.05$).

min (4 °C) to obtain precipitates using a Beckman ultracentrifuge (S.A.). The lipid from the precipitate was extracted with 2 mL of $CHCl_3$/MeOH (2:1, v/v) according to a conventional method.

Analysis of Composition of Fatty Acids. The lipids were methylated with 20% tetramethylguanidine in methanol (100 °C, 10 min), followed by an additional reaction with 1.0 N H_2SO_4/methanol (55 °C, 5 min) (*22*). The composition of the fatty acid profile was analyzed by GC (Hewlett-Packard 5890, Little Fall, TX) equipped with FID and a fused silica capillary column, Supelcowax-10 (60 m × 0.32 mm, i.d., 25 μm film thickness, Bellefonte, PA). The oven temperature was increased from 180 to 200 °C at a rate of 2 °C/min and was 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 by comparison with the relative retention times of the authentic standards and synthetic CLA using heptadecanoic acid (C17:0) as an internal standard.

Statistical Analysis. Data are presented as mean ± SD. Data were analyzed by one-way ANOVA using the Statistical Analysis System (*23*). Differences were considered to be significant at $p < 0.05$.

RESULTS

Inhibition of Cell Growth. Figure 1A shows the inhibitory effect of *t,t* CLA on the growth of MCF-7 cells, determined by incubating the cells in serum-free medium with various concentrations of *t,t* CLA (5, 10, 20, 40, and 60 μM) for 6 days. The growth of cells incubated with *t,t* CLA was effectively inhibited, whereas control cells proliferated rapidly for 6 days. The growth inhibitory effect increased with increasing concentrations of *t,t* CLA, but concentrations higher than 40 μM did not significantly improve growth inhibition during the entire period of incubation time. In addition, the growth of cells treated with 40 μM *t,t* CLA was significantly inhibited ($p < 0.05$) on the fourth day of incubation relative to the second day of incubation, but was not further inhibited on the sixth day of incubation.

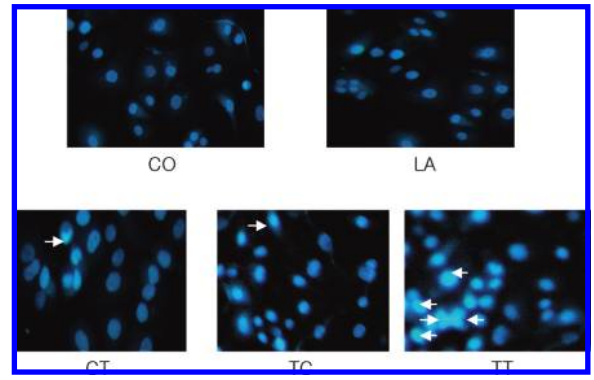

Figure 2. Hoechst 33258 staining of MCF-7 cells treated with 40 μM *t,t* CLA for 4 days. CO, LA, CT, TC, and TT represent control cells and linoleic acid, *c*9,*t*11 CLA, *t*10,*c*12 CLA, and *t,t* CLA treated cells, respectively. Fluorescence microscopic analysis of cells showed morphological changes of the nuclei. Arrows indicate fragmented or condensed nuclei.

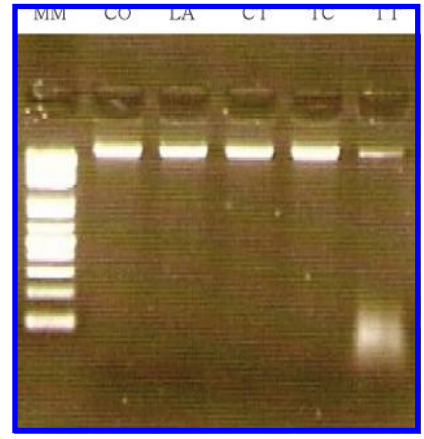

Figure 3. Agarose gel electrophoresis pattern of DNA from MCF-7 cells treated with 40 μM *t,t* CLA for 4 days. CO, LA, CT, TC, and TT lanes represent control cells and linoleic acid, *c*9,*t*11 CLA, *t*10,*c*12 CLA, and *t,t* CLA treated cells, respectively. Lane MM indicates molecular markers. The electrophoresis pattern of DNA was visualized under UV light.

The efficacy of the growth inhibition by 40 μM of *t,t* CLA was subsequently compared in relation to that by *t*10,*c*12 CLA, *c*9,*t*11 CLA, and linoleic acid (**Figure 1B**). On the fourth day of incubation, the growth rate following *t,t* CLA treatment was 22%, whereas the growth rates following *c*9,*t*11 CLA and *t*10,*c*12 CLA treatment were 54 and 48%, respectively. Although both the *t*10,*c*12 isomer and the *c*9,*t*11 CLA isomer inhibited the proliferation of cells, *t,t* CLA showed the strongest inhibitory effect ($p < 0.05$) during the entire period of culturing. Thus, *t,t* CLA exhibits the most potent cytotoxic effect in MCF-7 cells among all of the CLA isomers tested.

Induction of Cell Apoptosis. To characterize the mechanism of cell death by *t,t* CLA, cytological alterations and DNA fragmentation, which are the biochemical hallmarks of apoptosis (*20*), were analyzed in MCF-7 cells cultured with 40 μM *t,t* CLA for 4 days, with reference to *c*9,*t*11 CLA, *t*10,*c*12 CLA, and linoleic acid (**Figures 2** and **3**). As shown in **Figure 2**, MCF-7 cells treated with *t,t* CLA were accompanied by nuclear condensation, determined as the brightness measured by fluorescent microscopy of the stained cells. Although a few cells

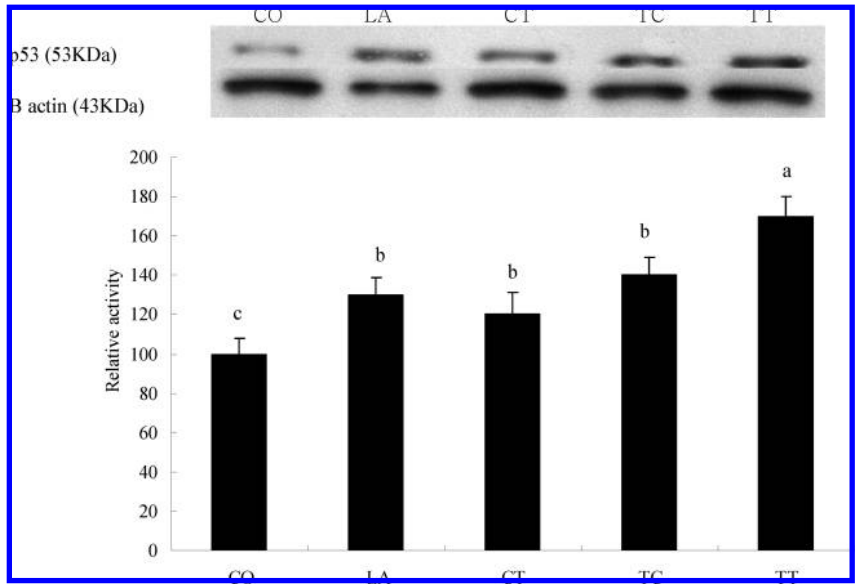

Figure 4. Expression of p53 in MCF-7 cells treated with 40 μM *t,t* CLA for 4 days. CO, LA, CT, TC, and TT represent control cells and linoleic acid, *c*9,*t*11 CLA, *t*10,*c*12 CLA, and *t,t* CLA treated cells, respectively. The band intensities relative to the control were quantified. Values are means ± SD ($n = 3$). Means with different lower case letters are significantly different at $p < 0.05$.

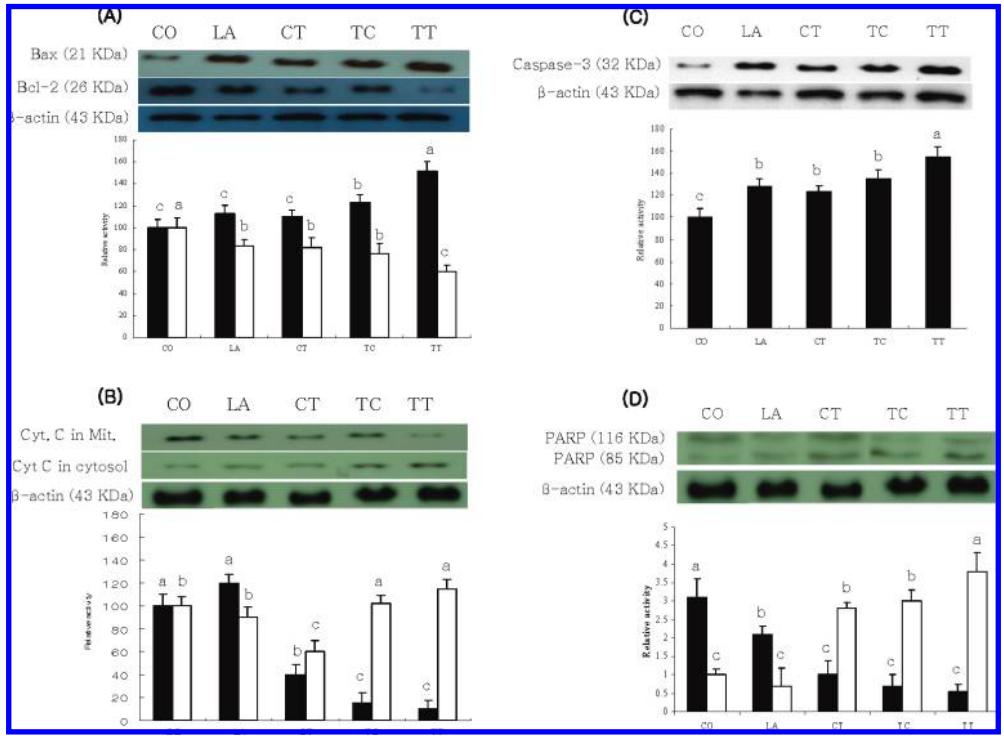

Figure 5. Western blot analyses of apoptotic proteins in MCF-7 cells treated with 40 μM *t,t* CLA for 4 days: (**A**) levels of Bax and Bcl-2 proteins; (**B**) levels of cytochrome *c* in the mitochondria and in the cytosol; (**C**) activity of caspase-3; (**D**) cleavage of PARP. CO, LA, CT, TC, and TT represent control cells and linoleic acid, *c*9,*t*11 CLA, *t*10,*c*12 CLA, and *t,t* CLA treated cells, respectively. The band intensities relative to the control were quantified. Values are means ± SD ($n = 3$). Means with different lower case letters are significantly different at $p < 0.05$.

were dead by *c*9,*t*11 CLA and *t*10,*c*12 CLA, the numbers of cells killed were lower than those killed by *t,t* CLA.

Such results were further supported by the chromosomal DNA fragmentation experiment. As shown in **Figure 3**, the characteristic DNA smear was more evident in cells treated with *t,t* CLA than in cells treated with *c*9,*t*11 CLA, *t*10,*c*12 CLA, and linoleic acid. No DNA ladder pattern was seen in any of the cells treated with *t,t* CLA and other types of CLA.

Modulation of Apoptotic Proteins. Using Western blotting, the quantities of p53, Bax, Bcl-2, cytochrome *c*, and PARP and the activity of caspase-3 were determined from MCF-7 cells treated with 40 μM *t,t* CLA for 4 days. These values were also compared in relation to *t*10,*c*12 CLA, *c*9, *t*11 CLA, and linoleic acid (**Figures 4** and **5**). The p53 tumor suppressor protein was significantly increased ($p < 0.05$) in *t,t* CLA treated cells compared to cells treated with other CLA isomers and also compared to the control (**Figure 4**). The expression of p53 protein was also increased by *t*10,*c*12 CLA, *c*9,*t*11 CLA, and linoleic acid treated cells, as compared to control cells, but was lower than that in *t,t* CLA treated cells.

Table 1. Major Fatty Acid Composition of Plasma Membrane in MCF-7 Cells Incubated with 40 μM CLA

fatty acid[b]	treatment[a]				
	CO	LA	CT	TC	TT
myristic acid (14:0)	3.4 ± 1.0 b	0.6 ± 0.2 c	8.2 ± 1.3 a	1.4 ± 0.5 c	1.0 ± 0.6 c
palmitic acid (16:0)	9.5 ± 1.5 a	11.8 ± 2.5 a	1.9 ± 0.8 c	2.4 ± 1.6 c	5.5 ± 3.5 b
stearic acid (18:0)	26.9 ± 2.8 a	5.9 ± 1.9 c	4.8 ± 2.2 c	9.1 ± 2.5 bc	13.9 ± 3.5 b
oleic acid (18:1)	23.5 ± 3.2 a	21.9 ± 6.3 a	18.5 ± 3.5 a	12.5 ± 3.2 b	22.5 ± 3.9 a
linoleic acid (18:2)	11.5 ± 0.9 b	45.9 ± 4.3 a	6.9 ± 1.5 c	2.8 ± 1.2 d	2.3 ± 2.1 d
α-linolenic acid (18:3)	9.9 ± 1.5 a	3.5 ± 1.5 bc	3.9 ± 2.5 bc	1.4 ± 0.8 c	4.4 ± 2.0 b
*c*9,*t*11 CLA	nd[c]	nd	40.5 ± 3.8 a	13.5 ± 2.5 b	10.9 ± 3.1 b
*t*10,*c*12 CLA	nd	nd	4.0 ± 1.2	45.9 ± 4.5	nd
t,*t* CLA	nd	nd	nd	nd	31.5 ± 2.6
arachidonic acid (20:4)	10.9 ± 1.8 a	7.2 ± 1.2 bc	6.5 ± 0.9 bc	7.9 ± 0.7 b	5.5 ± 0.5 c

[a] CO, LA, CT, TC, and TT represent control cells and linoleic acid, *c*9,*t*11 CLA, *t*10,*c*12 CLA, and *t*,*t* CLA treated cells, respectively. Results are expressed as means ± SD of three experimental data. Means with different letters in the same row are significantly different, $p < 0.05$. [b] Fatty acid is expressed as the area percentage of a given fatty acid to total fatty acids of interest. [c] nd, not detected.

As shown in **Figure 5A**, the expression of Bax protein was significantly increased ($p < 0.05$) in the *t*,*t* CLA treated cells, relative to that in the control cells, *c*9,*t*11 CLA treated cells, and linoleic acid treated cells. Meanwhile, Bcl-2 protein expression in cells treated with *t*,*t* CLA was significantly reduced ($p < 0.05$) compared to cells treated with *t*10,*c*12 CLA and *c*9,*t*11 CLA and control cells. These results suggest that the antiproliferative effect and apoptosis observed in the MCF-7 cells as a result of the *t*,*t* CLA treatment, relative to that of the *t*10,*c*12 CLA or *c*9,*t*11 CLA isomer, were directly related to the reciprocal expression of these two proteins.

Figure 5B shows the release of cytochrome *c* from the internal space of the mitochondria into the cytoplasm of *t*,*t* CLA treated cells. As compared to cells treated with other types of CLA isomers, the *t*,*t* CLA treated cells released a significant level of cytochrome *c* in the cytosol, with which the relative ratio of cytochrome *c* in cytoplasm to mitochondria is 6.78. Similar results, but lower efficacy, in the *t*10,*c*12 CLA treated cells and in the *c*9,*t*11 CLA treated cells were observed, with relative release ratios of 3.63 and 5.05, respectively. Moreover, a small amount of cytochrome *c* was released in the linoleic acid treated cells, with a relative release ratio of 0.30, as well as in the control cells, with a relative release ratio of 0.36.

The release of cytochrome *c* is believed to be followed by the activation of caspases. Thus, the activity of caspase-3 was investigated in the cells treated with *t*,*t* CLA (**Figure 5C**). The activity of caspase-3 in the *t*,*t* CLA treated cells was significantly ($p < 0.05$) higher than that in *t*10,*c*12 CLA, *c*9,*t*11 CLA, and linoleic acid treated cells. **Figure 5D** shows degradation of PARP in the *t*,*t* CLA treated cells. PARP is an intrinsic substrate for caspase-3 that would be cleaved during apoptosis in many different cell lines (*24*). The 85 kDa product appeared, together with a concomitant loss of 116 kDa full-length PARP, and the relative breakdown ratio of 85 to 116 kDa was 3.98. The breakdown ratio was 3.11 in the cells treated with *t*10,*c*12 CLA and, to a lesser extent, 2.11 in the cells treated with *c*9,*t*11 CLA. The relative breakdown ratio was 0.25 in the linoleic acid treated cells and 0.13 in the control cells.

Alteration of Membrane Fatty Acid Composition. The membrane fatty acid profile of the MCF-7 cells treated with 40 μM *t*,*t* CLA with reference to *c*9,*t*11 CLA, *t*10,*c*12 CLA, and linoleic acid for 4 days was analyzed and determine the effect of *t*,*t* CLA on the composition of membrane fatty acids (**Table 1**). The cells that had been treated with *t*,*t* CLA, *c*9,*t*11 CLA, *t*10,*c*12 CLA, and linoleic acid contained more *t*,*t* CLA, *c*9,*t*11 CLA, *t*10,*c*12 CLA, and linoleic acid, respectively, than the control cells. Thus, there was a concomitant decrease in the content of other fatty acids. The *t*,*t* CLA treated cells contained 31.5% *t*,*t* CLA, 10.9% *c*9,*t*11 CLA, and 2.3% linoleic acid. Similarly, the *t*10,*c*12 CLA treated cells contained 45.9% *t*10,*c*12 CLA, and 13.5% *c*9,*t*11 CLA, with 2.8% linoleic acid. In addition, the *c*9,*t*11 CLA treated cells possessed 40.5% *c*9,*t*11 CLA and 4.0% *t*10,*c*12 CLA, with a linoleic acid content of 6.9%. The composition of arachidonic acid was 5.5% in cells treated with *t*,*t* CLA, 6.5% in cells treated with *c*9,*t*11 CLA, 7.9% in cells treated with *t*10,*c*12 CLA, and 7.2% in cells treated with linoleic acid, whereas the arachidonic acid composition was 10.9% in control cells. These results indicate that the CLA isomers specifically reduced the composition of the linoleic and arachidonic acids in the membrane phospholipids. The presence of a substantial amount of other CLA isomers in cells treated with a given sample of CLA isomers might result from impurity of the CLA isomers in the CLA samples.

DISCUSSION

The present study clearly revealed that *t*,*t* CLA inhibited the growth of MCF-7 cells through the induction of apoptosis in conjunction with the modulation of arachidonic acid metabolism, which was superior to that of the *t*10,*c*12 CLA and *c*9,*t*11 CLA isomers. A number of studies have reported on apoptosis of rapidly dividing cancer cells containing *c*9,*t*11 CLA and *t*10,*c*12 CLA (*12–16*), but no reports on *t*,*t* CLA are available in the literature, with the exception of our previous paper (*16*). The molecular mechanism responsible for the antiproliferative action of *t*,*t* CLA is not clearly understood; however, the observations of the present study have clarified the apoptotic role of *t*,*t* CLA in regulating human breast MCF-7 cancer cells.

Evidence for the apoptosis of *t*,*t* CLA was observed by Hoechst 33258 staining (**Figure 2**) and DNA fragmentation analysis (**Figure 3**). The morphological features of apoptosis, such as the condensation since brightness and the fragmentation of the nucleus into discrete masses, were evident in the *t*,*t* CLA treated cells and, to a lesser extent, in the *t*10,*c*12 CLA treated cells, relative to the control cells (**Figure 2**), which showed a normal morphology in the cell nuclei. Such an event was already seen in osteosarcoma MG-63 cells treated with *t*,*t* CLA (*16*) and also in a variety of other cancer cells treated with *c*9,*t*11 CLA and *t*10,*c*12 CLA isomers (*12–16*). The DNA ladder pattern was not observed in MCF-7 cells treated with *t*,*t* CLA. However, as shown in **Figure 3**, the exposure of MCF-7 cells to *t*,*t* CLA caused a DNA fragmentation that is characteristic of apoptosis (*25*). We previously observed a distinct oligosomal ladder with different-sized DNA fragments in osteosarcoma MG-63 cells treated with *t*,*t* CLA, but not in osteosarcoma MG-63 cells treated with *c*9,*t*11 CLA and *t*10,*c*12 CLA isomers (*16*).

Kim et al. (*13*) also reported a distinct oligosomal ladder with a different-sized DNA fragment in Caco-2 cells treated with *t*10,*c*12 CLA. Thus, DNA fragmentation appeared to depend on the types of CLA isomers used and the type of cancer cell targeted.

It is evident that the apoptotic effect of *t,t* CLA on MCF-7 cells can be attributed to a pathway involving Bax- and Bcl-2-mediated mitochondrial dysfunctions. Because the p53 tumor suppressor protein was significantly increased by *t,t* CLA treated cells, as compared to *t*10,*c*12 CLA, *c*9,*t*11 CLA, and linoleic acid treated cells (**Figure 4**), it can be suggested that *t,t* CLA might have a stimulatory effect on the expression of p53 protein. The p53 protein, which plays a vital role in the induction of apoptosis in cancer cells (*26*), might reciprocally regulate the expressions of Bax and Bcl-2 proteins in response to *t,t* CLA, as shown in **Figure 3**. Compared to *c*9,*t*11 CLA and *t*10,*c*12 CLA treated cells, Bax protein expression was significantly higher ($p < 0.05$), whereas the Bcl-2 protein level was significantly lower ($p < 0.05$), in cells treated with *t,t* CLA (**Figure 5A**). Then, the Bax protein promoted the cytosolic release of cytochrome *c*, which, in turn, activated the key executioners of apoptosis, including the activation of caspase-3 and the cleavage of PARP (116 kDa) protein to yield the characteristic 85 kDa fragment. The release of cytochrome *c* from the mitochondria into the cytosol (**Figure 5B**), the expression of the caspase-3 protein (**Figure 5C**), and the proteolytic cleavage of the PARP protein (116 kDa) to the characteristic 85 kDa fragment (**Figure 5D**) in MCF-7 cells treated with *t,t* CLA were significantly higher relative to cells treated with *t*10,*c*12 CLA and linoleic acid cells. These results indicate that a mitochondrial damage dependent pathway might be involved in *t,t* CLA induced apoptosis in MCF-7 cells.

The antiproliferative effects of *t,t* CLA on MCF-7 cells through mitochondrial damage dependent apoptosis might be associated with the altered fatty acid profile of the cell membrane. Such a hypothesis is basically extracted from the finding that the growth inhibitory effect of CLA on cancer cell lines is related to the alteration of the cellular fatty acid composition and the regulation of the expressions of some genes (*8, 27*). The chemical structure of *t,t* CLA is similar to that of stearic acid, whereas the chemical structures of the *c*9, *t*11 and *t*10,*c*12 CLA isomers are similar to that of linoleic acid. Thus, as it is included in terms of the proportion of the *t,t* CLA to the saturated fatty acid composition, the portion of the unsaturated fatty acid composition induced by *t,t* CLA, which showed the most potent inhibitory effect on MCF-7 cells (**Figures 1** and **2**), was 23.1%, much lower than the 71.5% shown by *t*10,*c*12 CLA and the 61.8% shown by *c*9,*t*11 CLA (**Table 1**). Such changes in the fatty acid profile of the cell membranes might affect the expression of the p53, Bax, and Bcl-2 proteins in cells treated with CLA isomers; in our results, *t,t* CLA treatment induced an increase in p53 expression (**Figure 4**), a decrease in Bcl-2 expression, and an increase in Bax expression (**Figure 5A**). Increases in ROS and cytokine generation might be involved in the activation of apoptosis in MCF-7 cells, but we do not know the exact relationships. The down-regulation of p53 and the reciprocal regulation of Bax and Bcl-2 might be sufficient to shift the balance toward apoptosis in MCF-7 cells.

Interestingly, Park et al. (*28*) reported that the cytotoxicity of dietary CLA to colon cancer cells could be related to the ratio of Bax/Bcl-2 proteins, which functions as a modulator of cellular fate. Several other studies have suggested that increased Bax/Bcl-2 ratios could promote the susceptibility of cells to apoptosis (*13, 16*). The ratio of Bax/Bcl-2 in *t,t* CLA treated cells was significantly higher ($p < 0.05$) than that in *t*10,*c*12 CLA and *c*9,*t*11 CLAtreated cells, thus indicating the efficacy of the *t,t* CLA isomer in inducing apoptosis of human breast MCF-7 cancer cells.

On the other hand, the incorporation of *t,t* CLA in the membrane phospholipids also reduced the arachidonic acid content relative to that of *t*10,*c*12 CLA and *c*9,*t*11 CLA (**Table 1**), and this result may inhibit the biosynthesis of PGE_2 from arachidonic acid via the cyclooxygenase and prostaglandin H synthase pathways. Liu et al. (*27*) also reported that the *c*9,*t*11 and *t*10,*c*12 CLA isomers inhibit the formation of arachidonic acid and prostaglandin E_2 (PGE_2), which are involved in cell growth. Hence, in addition to the mitochondrial damage-dependent pathway involved in *t,t* CLA induced apoptosis in MCF-7 cells, an additional potential mechanism is a reduction in the synthesis of arachidonic acid and arachidonate-derived eicosanoids, which is associated with the stimulation of cancer cell growth. It is worth noticing that *t,t* CLA also might inhibit the activity and expression of phospholipase A2 (cPLA2) in MCF-7 cells that release arachidonic acid for eicosanoid production.

In conclusion, *t,t* CLA exhibited the most potent inhibitory effect on the growth of MCF-7 cells mediated by the induction of apoptosis, relative to *c*9,*t*11 CLA, *t*10,*c*12 CLA, and linoleic acid. The inhibitory effect of *t,t* CLA is mainly attributed to the pathway involving Bax- and Bcl-2-mediated mitochondrial dysfunctions. In addition, the inhibitory effect was, in part, attributed to the reduction of arachidonic acid content and its metabolism. Certainly, further investigations are warranted to explain the mechanisms of cell death induced by *t,t* CLA.

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