



Effects of Diglyceride-Conjugated Linoleic Acid on Proliferation and Differentiation of 3T3-L1 Cells

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Conjugated linoleic acid (CLA) has been recently reported to have an anti-obesity effect in animals and humans. The objective of this study was to investigate effects of diglyceride (DG)-CLA on proliferation and differentiation of 3T3-L1 preadipocytes. Cell proliferation was determined using WST-8 analysis and cell differentiation was determined by glycerol-3-phosphate dehydrogenase (GPDH) activity. Lipid accumulation in differentiating 3T3-L1 cells was determined by Oil red O staining. There were four experimental groups including vehicle control (DMSO), CLA, triglyceride (TG)-CLA, and DG-CLA. Treatments of CLA, TG-CLA, and DG-CLA at the concentrations of 10~1000 µg/ml reduced proliferation of preconfluent 3T3-L1 cells in a dose-dependent manner. Among them CLA was the most effective in the proliferation inhibition of preconfluent 3T3-L1 cells with increasing concentrations. Treatments of CLA and DG-CLA at the concentration of 100 µg/ml significantly inhibited differentiation of postconfluent 3T3-L1 cells as measured by GPDH activity ($p < 0.05$). In addition, treatments of CLA, TG-CLA, and DG-CLA effectively inhibited lipid accumulation during differentiation of 3T3-L1 cells. DG-CLA had the most inhibitory effect on the differentiation and lipid accumulation. These results suggest that the compounds including CLA have a respectable anti-obesity effect and that consumption of DG-CLA as a dietary oil may give a benefit for controlling overweight in humans.

Key words: Conjugated linoleic acid, Diglyceride, Glycerol-3-phosphate dehydrogenase, Obesity, Triglyceride, 3T3-L1 cells.

INTRODUCTION

Obesity is a major public health problem and a main cause of most of geriatric diseases in Western countries. It is associated with many health risks, including heart disease, diabetes mellitus, stroke, high blood pressure, gallbladder disease, and some forms of cancer (Kissebah and Krakower, 1994; Must *et al.*, 1999). According to a recent report from the National Health and Nutrition Examination Survey in the United States, it was estimated that over 65% of adults were overweight or obese, and 16% of children were overweight (National Center for Health Statistics, 2004; Flegal,

2005; Jeon *et al.*, 2006). Although diet, especially dietary fat, has been recognized as contributing to the development of obesity, differential effects have arisen with respect to individual fatty acids.

The physiological and anti-obesity effects of diglyceride (DG), which consists mainly of 1,3-DG, have been reported in numerous studies (Murata *et al.*, 1994; Taguchi *et al.*, 2001; Takatoshi *et al.*, 2002). A single dose of DG emulsion lowered the extent of increase in postprandial serum triglyceride (TG) levels in rats, compared with TG emulsion (Taguchi *et al.*, 2001). Dietary DG, in contrast to TG, decreased both body weight and visceral fat mass as determined by computed tomography in healthy men (Nagao *et al.*, 2000). In addition, dietary DG suppressed the accumulation of high-fat and high-sucrose diet-induced body fat in C57BL/6J mice (Murase *et al.*, 2001).

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Conjugated linoleic acid (CLA) refers to a group of isomers of linoleic acid (cis-9, cis-12 octadecadienoic acid). These isomers can either be positional (shifting of double bonds to 9-11 or 10-12 positions), geometric (cis/trans variations), or a combination of both. The major dietary source of CLA for humans is ruminant meats such as beef and lamb and dairy products including milk and cheese (DeLany *et al.*, 1999; Ostrowska *et al.*, 1999). CLA has been reported to be anticarcinogenic (Ip *et al.*, 1991; Chin *et al.*, 1992; Parodi, 1997) antiatherogenic, and immunomodulating agents (Lin *et al.*, 1995; Park *et al.*, 1999a). More recently, a crude mixture of CLA isomers has been shown to reduce body fat and enhance fat-free mass in animals and humans (Alfin-Stater *et al.*, 1968; Pariza *et al.*, 2001; Banni *et al.*, 2001). Although CLA has been also reported to inhibit proliferation and differentiation in 3T3-L1 preadipocyte (Park *et al.*, 1999b; Deckere *et al.*, 1999), there is also disagreement about CLA's effects on cellular differentiation. One study reported a stimulatory effect of CLA in murine 3T3-L1 preadipocytes (Satory and Smith, 1999). In addition, the treatment of CLA during adipocyte differentiation reduces lipid accumulation and inhibits the expression of PPAR- γ which is a nuclear receptor that activates genes involved in lipid storage and metabolism (Granlund *et al.*, 2005).

In the present study, we synthesized diglyceride (DG-CLA) from glycerol and CLA. The DG-CLA contained about 66% CLA as fatty acids. As 3T3-L1 cells are a reliable system for analyzing the development of adipocytes (MacDougald and Lane, 1995; Gregoire *et al.*, 1998), we chose this system to study the effect of DG-CLA during 3T3-L1 preadipocytes proliferation and differentiation. Although these cells are derived from mice, the basic mechanism for fat development appear to be similar in both rodent and human cells.

MATERIALS AND METHODS

Materials. Experimental materials including CLA,

Table 1. The composition of fatty acids in CLA, TG-CLA, and DG-CLA used in this study

	Amounts of fatty acids (%)						
	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	CLA	Linolenic acid	etc
CLA	6	3	12	2	74	2	1
TG-CLA	6	3	11	-	77	2	1
DG-CLA	6	3	13	7	66	3	2

The composition of fatty acids in test solutions was analyzed by gas chromatography. CLA, conjugated linoleic acid; TG, triglyceride; DG, diglyceride.

TG-CLA, and DG-CLA were obtained from Illshin wells Inc. (Cheongwon, Chungbuk). CLA typically produced for experimental purposes consisted of the cis-9, trans-11 and trans-10, cis-12 isomers (approximately a 50 : 50 ratio). The composition of fatty acids of the CLA, TG-CLA, and DG-CLA was analyzed by Gas-Liquid Chromatography (Table 1). The synthesis of DG-CLA was described in patent No. 10-0540875 (Illshin wells, Inc.).

3T3-L1 cell culture. 3T3-L1 preadipocytes derived from mouse fibroblast cell line were obtained from the Kore Research Institute of Bioscience and Biotechnology (KRIBB, Deajeon, Korea). 3T3-L1 cells were incubated in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% bovine calf serum (BCS; Gibco, NY, USA), 100 U/ml penicillin, and 100 g/ml streptomycin (Gibco, NY, USA) with 5% CO₂ at 37°C. The medium was renewed three times per week.

Differentiation of 3T3-L1 pre-adipocytes. 3T3-L1 preadipocytes were cultured for 24 h in a medium containing high-glucose DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, NY, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (adipocyte medium). After 24 h, the medium was substituted with high-glucose DMEM supplemented with 10% FBS, 10 μ g/ml insulin (Sigma-Aldrich, USA), 1 μ M dexamethasone (Sigma-Aldrich, USA), and 0.5 mM isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich) (Differentiation medium 1). After 48 h, the medium was substituted with DMEM, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10 μ g/ml insulin supplementation (Differentiation medium 2). After a 5-day induction period, the cells were incubated in the adipocyte medium with 5% CO₂ at 37°C. The medium was renewed three times per week.

Cell number assay. To assess proliferation of pre-confluent 3T3-L1 cells to the confluent cells, WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt] (Dojindo inc., U.S.A.) assay was performed. The cells were plated at density of 1×10^5 cells per ml into a 96-well microtiter-plate and allowed to attach overnight for 24 h. The cells were treated with various concentrations (10, 50, 100, 500, and 1000 μ g/ml) of CLA, TG-CLA, and DG-CLA and cultured for 24 h, and then analyzed using WST-8 assay. Briefly, in the 96 well microtiter-plate 10 μ l of WST-8 solution was added to each well containing 100 μ l medium and it was further incubated at 37°C for 2 h. The absorbance was recorded at 450 nm on microculture plate reader (Benchmark, Germany). The well containing only DMEM medium, 100 U/ml penicil-

lin, 100 mg/ml streptomycin, 0.1% DMSO, and WST-8 was used as the control. The optical density (OD) was measured and percent viability was calculated as OD of treated sample/OD of non-treated sample \times 100.

Glycerol-3-phosphate dehydrogenase activity assay. Differentiation of 3T3-L1 preadipocytes to adipocytes was determined using glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8) activity on incubation day 8. 3T3-L1 preadipocytes 1×10^5 cells per ml of the medium in a 96 well microtiter-plate were treated with 100 μ g/ml of CLA, TG-CLA, and DG-CLA for 48 h with 5% CO₂ at 37°C. The medium was changed and the cells were incubated with for 8 day. GPDH activity using a GPDH assay kit (Takara Bio, inc., Japan) was spectrophotometrically measured by absorbance at 340 nm. One unit was defined as the amount of enzyme required for consumption of 1 μ mol of NADH for one minute at 30°C. The GPDH activity (unit/ml) in the test samples was calculated from the following formula:

$$\text{GPDH activity (unit/ml)} = \frac{\Delta \text{OD}_{340} \times A \text{ (ml)} \times \text{Dilution ratio of the test sample}}{6.22 \times B \text{ (ml)} \times C \text{ (cm)}}$$

Δ OD₃₄₀: Decrease in the absorbance at 340 nm per minute

A (ml): Total reaction volume

B (ml): The volume of enzyme solution (diluted sample) added

C (cm): Optical path length of the cell used*

6.22: Millimolar absorption coefficient of NADH molecules

Oil red O staining. Intracellular lipid accumulation during differentiation of 3T3-L1 preadipocytes to adipocytes on day 8 was monitored by Oil red O staining. 3T3-L1 adipocytes 5×10^5 cells per ml with cover glasses in a 6-well plate were treated with 100 μ g/ml of CLA, TG-CLA, and DG-CLA for 48 h and the cells were incubated with 5% CO₂ at 37°C for 8 day. The cells on the cover glass were fixed with 10% formalin in phosphate buffer saline at pH 7.4 for 30 min at room temperature. After fixation, cells were washed once with PBS and stained with a filtered Oil red O (Sigma St, Louis, MO, U.S.A) stock solution (0.5 g Oil red O in 100 ml of 100% polypropylene glycol) for 15 min at room temperature. Then, the cells were washed twice with distilled water for 15 min and observed by a light microscope (Olympus, Tokyo, Japan).

Statistical analysis. Data were expressed as the mean \pm SEM. Statistical significance between the con-

trol group and treatment groups was determined by one-way analysis of variance (ANOVA), followed by the LSD, Turkey, Duncan test using the SPSS 10.0 statistic computer program. A difference at the level of $p < 0.05$ was considered to be statistically significant.

RESULTS

3T3-L1 Cell viability. Preconfluent 3T3-L1 cells were treated with various concentrations of CLA, TG-CLA, and DG-CLA and incubated with serum-free medium for 24 h. Cell viability was measured using WST-8 analysis and was expressed as % cell viability compared with the control. The cell viabilities by treatments with CLA, TG-CLA, and DG-CLA were shown in Fig. 1. The treatments of CLA, TG-CLA, and DG-CLA reduced proliferation of 3T3-L1 cells in a dose-dependent manner. At the concentration of 100 μ g/ml, the treatments of CLA, TG-CLA, and DG-CLA significantly inhibited the cell proliferation by about 25, 24, and 22%, respectively, compared with the control (Fig. 1).

Glycerol-3-phosphate dehydrogenase (GPDH) activity. Differentiation of 3T3-L1 preadipocytes treated with CLA, TG-CLA, and DG-CLA at the concentration of 100 μ g/ml was measured by GPDH activity. The GPDH enzyme activity for control (DMSO) was 3.20 ± 0.04 (U/ml). The treatments of CLA and DG-CLA at the concentration of 100 μ g/ml significantly decreased the GPDH activities by 0.91 and 1.11 (U/ml), respectively, com-

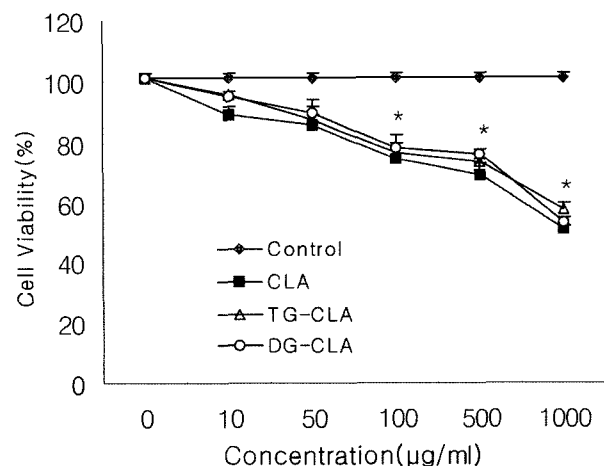


Fig. 1. Effect of conjugated linoleic acid (CLA), triglyceride-conjugated linoleic acid (TG-CLA), and diglyceride-conjugated linoleic acid (DG-CLA) on viability of 3T3-L1 cells. After 3T3-L1 cells were treated with various concentrations of test solutions for 24 h, cell viability (%) was measured using the WST-8 assay. Data were the means \pm SEM (n = 4). *Significantly different from the control ($p < 0.05$).

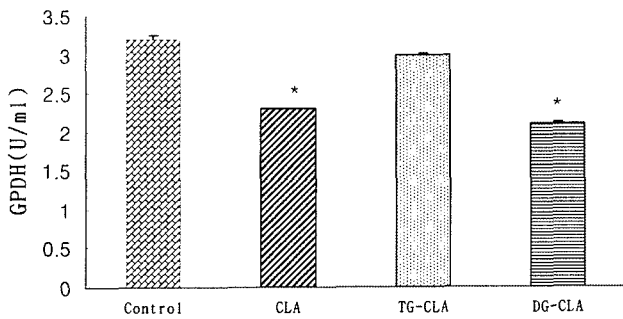


Fig. 2. Glycerol-3-phosphate dehydrogenase activity (GPDH) of 3T3-L1 cells treated with conjugated linoleic acid (CLA), triglyceride-conjugated linoleic acid (TG-CLA), and diglyceride-conjugated linoleic acid (DG-CLA). The cells were treated with test solutions at the concentration of 100 $\mu\text{g/ml}$ for 48 h and incubated with differentiation media for 8 days. The GPDH activity was determined using a commercial kit. The bars indicate mean \pm SEM ($n = 3$) *Significantly different from the control at $p < 0.05$.

pared with the control ($p < 0.05$). TG-CLA treatment also decreased the GPDH activity by 0.20 (U/ml) but

the decrease was not significantly different from the control (Fig. 2).

Lipid accumulation in adipocytes. After 3T3-L1 preadipocytes were treated with CLA, TG-CLA, and DG-CLA at the concentration 100 $\mu\text{g/ml}$ for 24 h, morphological changes and lipid accumulation during the cell differentiation were observed by Oil red O staining (Fig. 3). The 3T3-L1 preadipocytes treated with DMSO were completely differentiated to adipocytes after incubation with differentiation medium for 8 days (Fig. 3A). The lipid accumulation was clearly observed in the cytoplasm of the adipocytes (Fig. 3A). Treatments of CLA, TG-CLA, DG-CLA at the concentration of 100 $\mu\text{g/ml}$ inhibited the accumulation of lipid droplets in the cytoplasm of adipocytes (Fig. 3B, 3C & 3D). However, the inhibition of lipid accumulation by the treatment of DG-CLA was stronger than that by CLA or TG-CLA (Fig. 3).

DISCUSSION

The objective of the present study was to investigate

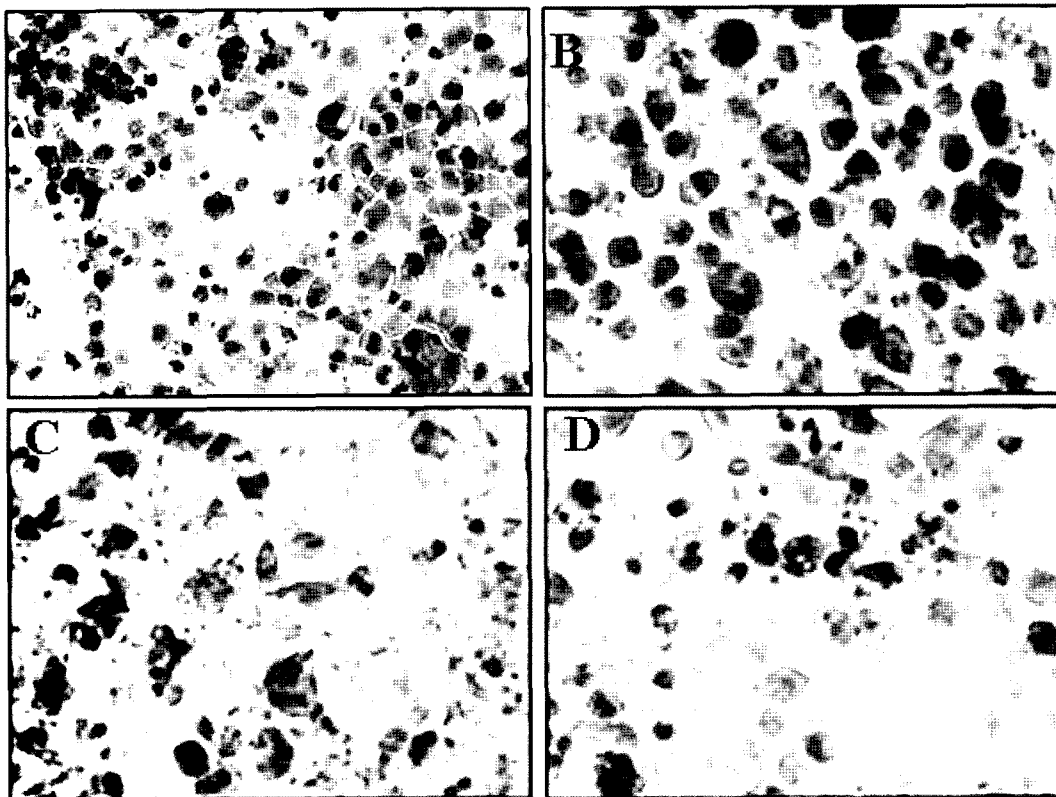


Fig. 3. Morphology of 3T3-L1 cells treated with conjugated linoleic acid (CLA), triglyceride-conjugated linoleic acid (TG-CLA), and diglyceride-conjugated linoleic acid (DG-CLA). The cells were treated with test solutions at the concentration of 100 $\mu\text{g/ml}$ for 48 h and incubated with differentiation media for 8 days. Lipid accumulation was observed with Oil red O staining. $\times 200$ magnification. A, Control (DMSO); B, CLA; C, TG-CLA; D, DG-CLA.

inhibitory effects of CLA, TG-CLA, and DG-CLA on proliferation and differentiation of 3T3-L1 preadipocytes. In *in vitro* cell system, the test solutions including CLA inhibited proliferation of preconfluent 3T3-L1 cells, the differentiation of postconfluent 3T3-L1 cells, and cytoplasmic lipid accumulation, as determined by WST-8 assay, GPDH activity, and Oil red O staining, respectively.

The inhibitory effects on proliferation and differentiation of 3T3-L1 cells in *in vitro* anti-obesity studies have been used as useful biomarkers for the well-known functions of CLA in many previous studies (Ha *et al.*, 1990; Lee *et al.*, 1994; Miller *et al.*, 1994; Pariza *et al.*, 2001; Takatoshi *et al.*, 2001; Terpstra *et al.*, 2003). In this study, CLA-containing compounds inhibited proliferation of preconfluent 3T3-L1 cells, which was consistent with other results (Brodie *et al.*, 1999). They reported that the stage of cell growth influenced the effect of CLA on cell number. Regardless of the medium, CLA treatment of preconfluent cells resulted in lower cell number, while postconfluent cells treated with CLA were unaffected (Brodie *et al.*, 1999).

CLA treatment inhibited the differentiation of 3T3-L1 preadipocytes into adipocyte as indicated by reduction of GPDH activity and lipogenesis as observed by Oil red O staining (Brodie *et al.*, 1999; Kang *et al.*, 2003). DG has been also reported to have anti-obesity effects in numerous studies (Taguchi *et al.*, 2001; Takatoshi *et al.*, 2002). DG decreased body weight, visceral fat, and serum TG levels (Murata *et al.*, 1994; Nagao *et al.*, 2000; Murase *et al.*, 2001). In our study, the CLA treatment effectively inhibited differentiation of 3T3-L1 preadipocytes and lipid accumulation in the cytoplasm of 3T3-L1 cells. The DG-CLA synthesized from glycerol and CLA, which contained 66% CLA as fatty acids, had the most effective inhibition on differentiation of 3T3-L1 preadipocytes and lipid accumulation during differentiation of 3T3-L1 preadipocytes. Our data showed that when preadipocytes were triggered to differentiation, CLA or CLA-containing compounds (TG-CLA and DG-CLA) significantly diminished the GPDH activity and markedly reduced the proportion of newly formed adipocytes. At the concentration of 100 $\mu\text{g/ml}$, the decreases in GPDH activity by treatments of CLA and DG-CLA were 0.91 and 1.11 (U/ml), respectively, indicating that DG-CLA had the stronger effect than CLA alone. The reduction of 3T3-L1 cells differentiation might be due to the inhibitory action by CLA on fatty acid synthase (FAS) activity (Brodie *et al.*, 1999; Liu *et al.*, 2004).

An inhibitory effect by DG-CLA on lipid accumulation in adipocytes indicates that the treatment might play an important role with regard to lipid filling during lipogene-

sis. The lipid accumulation in the cytoplasm of adipocytes can be determined by balance of the lipogenesis and lipolysis. Our results indicate that CLA and CLA-containing compounds suppressed adipocyte differentiation and reduced lipid accumulation during adipocyte differentiation, which may have the potential to produce anti-obesity effects in animals and humans (Kim *et al.*, 2000; Liu *et al.*, 2004; Granlund *et al.*, 2005). Recently, dietary CLA has been shown to reduce body fat mass in various experimental animals lean/obese mice (Park *et al.*, 1997; West *et al.*, 1998; Park *et al.*, 1999a, b; Delany *et al.*, 1999; Tsuboyama-Kasaoka *et al.*, 2000; Miner *et al.*, 2001; Ohnuki *et al.*, 2001; Peters *et al.*, 2001). It is known that the expression of hepatic PPAR- γ is increased in some obese and diabetic model mice (Burant *et al.*, 1997). A down-regulation of these genes might be correlated with the subsequent attenuation of lipid accumulation. CLA was known to decrease the PPAR- γ expression in 3T3-L1 cells (Kang *et al.*, 2003). In the present study, although we did not determine the PPAR- γ expression in 3T3-L1 cells, the inhibitory effect of preadipocyte differentiation or adipogenesis by CLA or CLA-containing compounds might be related to prevention of lipid accumulation in cytoplasm of 3T3-L1 cells, probably due to acting PPAR- γ modulator.

In conclusion, the CLA TG-CLA, and DG-CLA effectively inhibited differentiation of 3T3-L1 cells and the inhibitory effectiveness by DG-CLA was the strongest among them. Further studies should be necessary to illustrate the detail mechanisms of DG-CLA on inhibition of differentiation and lipid accumulation in 3T3-L1 cells.

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