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Comparison of Diglyceride, Conjugated Linoleic Acid, and Diglyceride-Conjugated Linoleic Acid on Proliferation and Differentiation of 3T3-L1 Preadipocytes

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ABSTRACT – Conjugated linoleic acid (CLA) reduces fat deposition in several mammalian species. The proposed mechanisms for this effect are reduced preadipocyte proliferation and differentiation. The objective of this study was to investigate the inhibitory effects of diglyceride (DG), CLA, DG-CLA of proliferation and differentiation of 3T3-L1 preadipocytes. Cell viability 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 measured by Oil red O staining. The proliferation of preconfluent 3T3-L1 cells by treatments of DG, CLA, and DG-CLA was reduced in a dose-dependent manner. CLA among them was the most effective in reduction of viable cells with increasing concentrations. Treatments of the DG, CLA, and DG-CLA at the concentration of 100 •Ïg/ml for 48 h significantly inhibited differentiation of 3T3-L1 cells ($p<0.05$). In addition, cytoplasmic lipid accumulation during differentiation of the 3T3-L1 preadipocytes was also inhibited by treatments of the test solutions. DG-CLA was the most effective in the inhibition of differentiation and lipid accumulation in 3T3-L1 cells. These results indicate that the DG including CLA as fatty acids is more effective for anti-obesity than DG or CLA alone and that consumption of DG-CLA as a dietary oil may give a benefit for controlling overweight in humans.

Key Words: conjugated linoleic acid, differentiation, diglyceride, obesity, proliferation, 3T3-L1 cells

Introduction

Obesity is a major public health problem and main cause of most of geriatric diseases in Western countries. Although diet, expecially dietary fat, has been recognized as contributing to the development of obesity, differential effects have arisen with respect to individual fatty acids.

Conjugated linoleic acid (CLA) is the collective acronym for combinations of positional and geometric isomers of linoleic acid that exist naturally in dairy products and meats[1,2]. It is produced in ruminant animals via biohydrogenation of polyunsaturated fatty acids as well as during the mechanical processing of dairy products[3,4]. CLA has proven to be anti-carcinogenic[3,5,6], antiatherogenic, and immunemodulating agents[7,8]. The anticarcinogenic effect of CLA was originally proposed to be mediated by its antioxidative properties[5,9]. More recently, a crude mixture of CLA isomers has been shown to reduce body fat and enhance fat-free mass in animals and humans[10-12]. Mechanisms that have been proposed to explain the decreased fat deposition include decreased preadipocyte proliferation and differentiation, as demonstrated in the rodent-derived clonal cell line, 3T3-L1 cells[13-14]. Although CLA has been also reported to inhibit proliferation and differentiation in 3T3-L1 preadipocyte, there is also disagreement about CLA's effects on cellular differentiation. One study reported a stimulatory effect of CLA in murine 3T3-L1 preadipocytes[4]. 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[15].

Meanwhile, the physiological and anti-obesity effects of

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diglyceride (DG), which consists mainly of 1,3-DG, have been reported in numerous studies[16-18]. A single dose of DG emulsion lowered the extent of increase in postprandial serum triglyceride (TG) levels in rats, compared with TG emulsion[17]. Dietary DG, in contrast to TG, decreased both body weight and visceral fat mass as determined by computed tomography in healthy men[19]. In addition, dietary DG suppresses the accumulation of high-fat and high-sucrose diet-induced body fat in C57BL/6J mice[20].

In the present study, DG from soybean oil, CLA (purity, 77%), and DG-CLA containing 22% CLA as fatty acids were compared for antiobesity effect in 3T3-L1 cells. As 3T3-L1 cells are a reliable system for analyzing the development of adipocytes[21,22], we chose this system to study the effect of DG-CLA during 3T3-L1 preadipocytes proliferation and differentiation. Although these cells are derived form mice, the basic mechanism for fat development appear to be similar in both rodent and human cells.

Materials and Methods

Materials

Experimental materials including DG, CLA, and DG-CLA were obtained from the Illshin wells Inc. (Cheongwon, Chungbuk). CLA typically produced for experimental purposes was composed of the cis-9, trans-11 and trans-10,cis-12 isomers (approximately a 50:50 ratio). The composition of fatty acids of DG, CLA, and DG-CLA was analyzed by Gas-Liquid Chromatography (Table 1). The DG-CLA was produced following to 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% CO_2 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, 100 mg/ml streptomycin (adipocyte medium). After 24 h, the medium was substituted with high-glucose DMEM supplemented with 10% FBS, 10 μg/ml insulin (Sigma-Aldrich, USA), 1 μM 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 μg/ml insulin supplementation (Differentiation medium 2). After a 5-day induction period, the cells were incubated in the adipocyte medium with 5% CO_2 at 37°C. The medium was renewed three times per week.

Proliferation (viability) assay

To assess proliferation of preconfluent 3T3-L1 cells to the fibroblast cells, WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, moonosodium salt] (Dojindo inc., U.S.A) assay was performed. The cells were plated at density of 1×10 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 μg/ml) of DG, CLA, and DG-CLA and cultured for 24 h, and then analyzed using WST-8 assay. Briefly, in the 96 well microtiter-plate 10 μl of WST-8 solution was added to each well containing 100 μl 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 penicillin, 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 ×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 cells per ml of the medium in a 96 well microtiter-plate were treated with 100 μg/ml of DG, CLA, and DG-CLA for 48 h with 5% CO_2 at 37°C. The medium was

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

	Amounts of fatty acids (%)						
	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	CLA	Linolenic acid	etc
DG	3	3	31	56	-	4	4
CLA	6	3	12	2	74	2	1
DG-CLA	3	2	24	43	22	3	3

The composition of fatty acids was analyzed by gas chromatography. DG: diglyceride, CLA: conjugated linoleic acid.

changed and the cells were incubated with for 8 day. GPDH activity using a GPDH assay kit (Takara Bio, inc., Japan) was spectrophometrically determined as measured by absorbance at 340 nm. One unit was defined as the amount of enzyme required for consumption of 1 μmol of NADH for one minute at 30°C. The GPDH activity (unit/ml) in the test samples was calculated from the following formula:

$$\text{GPDH activity (unit/ml)} = \frac{OD340 \times A(ml) \times \text{Dilution ratio of the test sample}}{6.22 \times B(ml) \times C(cm)}$$

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 cells per ml with cover glasses in a 6 well plate were treated with 100 μg/ml of DG, CLA, and DG-CLA for 48 h and the cells were incubated with 5% CO_2 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% polypylene 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 ± SEM. Statistical significance between the control 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 proliferation

Preconfluent 3T3-L1 cells were treated with various concentrations of DG, CLA, and DG-CLA and incubated with serum-free medium for 24 h. Cell viability was determined using WST-8 analysis and was expressed as % cell viability compared with the vehicle control. The cell viabilities by treatments with DG, CLA, and DG-CLA were shown in Fig. 1. The treatments of DG, CLA, and DG-CLA reduced the proliferation of preconfluent 3T3-L1 cells in a dose-dependent manner. At the concentration of 100 μg/ml, the treatments of DG, CLA, and DG-CLA significantly ($p<0.05$) inhibited the cell proliferation by about 24, 25, and 22%, respectively (Fig. 1).

Glycerol-3-phosphate dehydrogenase (GPDH) activity

DG, CLA, and DG-CLA at the concentration of 100 μg/ml were treated to postconfluent 3T3-L1 cells and the cell differentiation was measured by GPDH enzyme activity (Fig. 2). At the concentration of 100 μg/ml, the GPDH enzyme activity of the control was 3.20 ± 0.04 (U/ml). The treatments of DG, CLA and DG-CLA significantly decreased the GPDH activities by 0.55, 0.91 and 0.97 (U/ml), respectively, compared with the control ($p<0.05$). The inhibitory effect of DG-CLA was the strongest among test solutions (Fig. 2).

Lipid accumulation during differentiation

After 3T3-L1 preadipocytes were exposed to DG, CLA and DG-CLA at the concentration 100 μg/ml for 24 h, morphological changes and lipid accumulation in the cells 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). Adipocytes of control group showed lipid accumulation in the cytoplasm. Treatments of

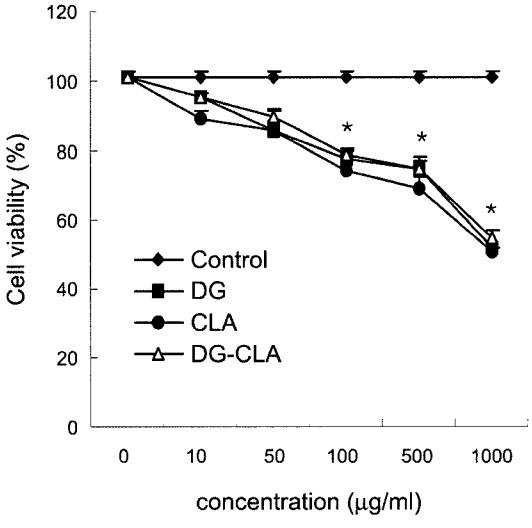

Fig. 1. Effect of diglyceride (DG), conjugated linoleic acid (CLA), and diglyceride-conjugated linoleic acid (DG-CLA) on viability of 3T3-L1 cells. After 3T3-L1 cells were treated with various concentrations of test solutions for 24 h, cell viability (%) was measured using the WST-8 assay. Data were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

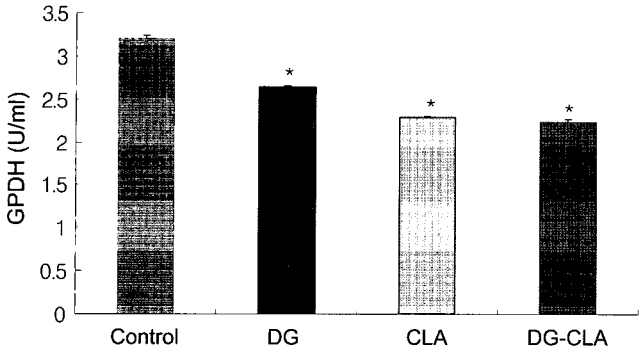

Fig. 2. Glycerol-3-phosphate dehydrogenase activity (GPDH) of 3T3-L1 cells treated with diglyceride (DG), conjugated linoleic acid (CLA), and diglyceride-conjugated linoleic acid (DG-CLA). The cells were treated with test solutions at the concentration of 100 μg/ml for 48 h and incubated with differentiation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

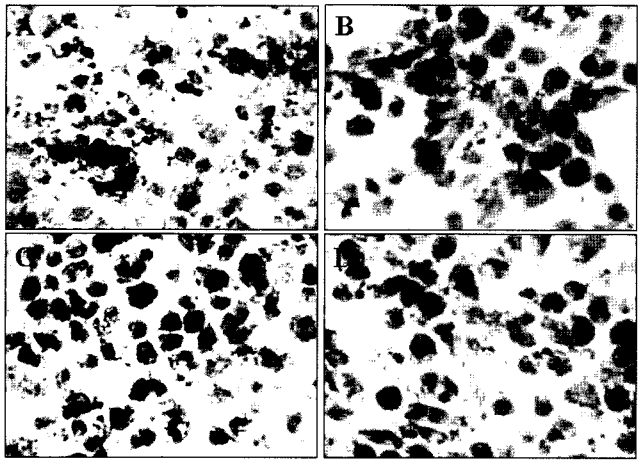

Fig. 3. Morphology of 3T3-L1 cells treated with diglyceride (DG), conjugated linoleic acid (CLA), and diglyceride-conjugated linoleic acid (DG-CLA). The cells were treated with test solutions at the concentration of 100 μg/ml for 48 h and incubated with differentiation media for 8 days. Lipid accumulation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

DG, CLA, and DG-CLA at the concentration of 100 μg/ml effectively reduced the accumulated lipid droplets in the cytoplasm of adipocytes (Fig 3B-D). The inhibitory effect of DG-CLA for lipid accumulation in the cytoplasm of adipocytes was slightly stronger than that of DG or CLA treatment (Fig. 3).

Discussion

We investigated the inhibitory effects of DG, CLA, and DG-CLA on proliferation and differentiation of 3T3-L1 cells. The DG, CLA, and DG-CLA inhibited the proliferation, differentiation, and lipid accumulation in 3T3-L1 cells, as determined by WST-8 assay, GPDH activity, and Oil red O staining, respectively. The inhibition by DG-CLA was the most effective although it did not have a synergistic effect from DG and CLA.

The inhibitory effects on proliferation and differentiation of 3T3-L1 cells have been used as useful biomarkers for the well-known functions of CLA in many previous cell studies[9,11,23-26]. In this study, CLA inhibited proliferation of preconfluent 3T3-L1 cells, which was consistent with other results[27]. 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[27]. Although DG-CLA contained only 22% CLA as fatty acids, it also inhibited the cell proliferation in the present study. The effects of CLA and DG-CLA were similar regardless of the amounts of CLA.

The CLA treatment inhibited the differentiation of 3T3-L1 preadipocytes into adipocytes as indicated by reduction of GPDH activity and lipogenesis as observed by Oil red O staining[27,28]. DG has been also reported to have anti-obesity effects in numerous studies[17,18]. DG decreased body weight, visceral fat, and serum TG levels[16,19,20]. In our study, CLA treatment effectively inhibited differentiation of 3T3-L1 preadipocytes and lipid accumulation in *in vitro* cell system. The DG-CLA containing 22% CLA had the most effective inhibition on differentiation of 3T3-L1 cells and lipid accumulation during differentiation of the cells. Our data showed that when preadipocytes were triggered to differentiation, DG, CLA or DG-CLA at the concentration of 100 μg/ml significantly diminished GPDH activity by 17.2%, 28.4% and 30.3% respectively, compared to the control, indicating that DG-CLA had the stronger effect than DG or CLA alone. The reduction of 3T3-L1 cells differentiation might be due to the inhibitory action by CLA on fatty acid synthase (FAS) activity[27,29]. The inhibitory effect by DG-CLA on lipid accumulation in adipocytes indicates that DG-CLA might play an important role with regard to lipid filling during lipogenesis. The lipid accumulation in the cytoplasm of adipocytes can be determined by balance of the lipogenesis and lipolysis. Our results indicate that CLA and DG-CLA suppressed adipocytes differentiation and reduced lipid accumulation during adipocytes differentiation, which may have the potential to produce anti-obesity effects in animals and humans[15,29,30]. Recently, dietary CLA has been shown to reduce body fat mass in various experimental animals lean/obese mice[1,8,13,31-36]. It is known that the expression of hepatic PPAR-γ is increased in some obese and diabetic model mice[37]. 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[28]. In the present study, although we did not determined the PPAR-γ expression in 3T3-L1 cells, the inhibitory effect of preadipocyte differentiation or adipogenesis by CLA or DG-CLA compounds may be related to prevention of lipid accumulation in cytoplasm of 3T3-L1 cells, probably due to acting PPAR-γ modulator.

In conclusion, the DG, CLA and DG-CLA effectively inhibited differentiation of 3T3-L1 cells and the inhibitory effectiveness by DG-CLA was the stronger than CLA alone in spite of the smaller amounts of CLA. Further studies should be necessary to illustrate the detail mechanisms of DG-CLA on inhibition of preadipocyte differentiation and lipid accumulation in 3T3-L1 cells.

요 약

Conjugated linoleic acid (CLA)는 전지방세포의 증식 및 분화를 억제시키므로서 포유동물에서 지방의 축적을 감소시키는 것으로 알려져 있다. 논 연구의 목적은 diglyceride (DG), CLA, 및 DG-CLA가 전지방세포의 증식 및 분화를 억제시키는지를 알아보고자, 세포생존측정, glycerol-3-phosphate dehydrogenase (GPDH) 활성측정, 지방축적에 대한 Oil red O 염색을 실시하였다. DG, CLA, 및 DG-CLA의 처치는 전지방세포의 증식을 농도 의존적으로 감소시켰으며, 그러한 효과는 CLA에서 가장 강하게 나타났다. 이들 시험물질의 100 μg/ml 농도에서 DG, CLA, DG-CLA는 유의적으로 GPDH의 활성을 낮추었으며($p<0.05$), 이러한 결과는 전지방세포의 분화를 억제하는 것을 의미한다. 더불어 이들 시험물질은 전지방세포의 분화과정 중에 지방축적을 효과적으로 억제시켰다. 더욱이 DG-CLA가 DG 혹은 CLA보다 분화 및 지방축적에 대해 더 강한 효과를 보였다. 이러한 결과로부터 DG-CLA의 섭취는 사람에서 체중조절의 유용한 효과를 얻을 수 있을 것이다.

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