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Cytotoxic effects of the conjugated linoleic acid isomers t10c12, c9t11-CLA and mixed form on rat hepatic stellate cells and CCl_4-induced hepatic fibrosis☆

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Abstract

Rat hepatic stellate cells (HSC-T6) were incubated for 24 h with 10–180 μM of t10c12 (98%), c9t11 (96%) and a mixed form (c9,t11:t10,c12; 41%:44%) of conjugated linoleic acid (CLA). The MTS dye reduction was measured to verify cell viability in a dose-dependent manner. Among the three CLAs, c9,t11-CLA exhibited the most intense cytotoxic effect on HSCs, the survival rate of which was reduced to 60% under 80 μM of treatment, while cell survival was slightly affected by the mixed form. Three CLA-induced cell deaths were determined by measuring DNA fragmentation using 4′,6-diamidino-2-phenylindole staining. The degrees of DNA fragmentation were the most severe in HSC treated with 80 μM of c9,t11-CLA. The mitogen-activated protein kinase/extracellular signal-regulated kinase-kinase and mitogen-activated or extracellular signal-regulated protein kinase (MEK) 1 and 2 were not activated in the t10,c12-CLA treatment. This suggests that the MEK-dependent apoptosis signal is crucial in HSC, which is induced by c9,t11 and mixed CLA. In order to evaluate the protective effect of CLA on carbon tetrachloride (CCl_4)-induced hepatic fibrosis in vivo, animals were treated with 10% CCl_4 to induce hepatic fibrosis during all experimental periods. Rats were divided into two treatment groups: (1) control diet with tap water ad libitum ($n=15$) and (2) 1% CLA diet with tap water ad libitum ($n=15$). In the CLA-supplemented rat livers, α-smooth muscle actin-positive cells were significantly reduced around the portal vein. In addition, collagen fibers were not detected in the CLA-treated group. These results suggest that 9c,11t-CLA influences cytotoxic effect on HSC in an MEK-dependent manner and preserving liver from fibrosis.

Keywords: Conjugated linoleic acid; Hepatic stellate cell; MAPK pathway; Hepatic fibrosis

1. Introduction

Conjugated linoleic acid (CLA) refers to a mixture of polyunsaturated fatty acids that exist as stereoisomers of conjugated dienoic octadecadienoic acid (18:2). CLA is found in natural sources such as meat (2.9–5.6 mg CLA per gram of fat) from ruminants and in dairy products [1–3]. In animal studies, CLA has provided several beneficial effects, as it is an anticarcinogenic, it is antiatherosclerosic and it can decrease body weight and the fat pad [4–14]. CLA was initially identified as an anticarcinogenic agent [1], and it has been identified as a method to prevent cancer in several animal models, as well as in humans, which includes breast cancer and prostate cancer cells [15], colorectal and prostatic cancer cells [16–18] and colon tumors [19–21]. Most CLA studies have been performed on a mixture of isomers, which contain t10,c12 and c9,t11-CLA as the major compounds.

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At present, purified isomers are commercially available, and the differences in cytotoxicity and cellular mechanisms between isomers have been extensively studied.

Yamasaki et al., however, have recently reported that t10,c12 CLA has a potent cytotoxic effect on rat hepatoma dRLh-84 cells via an apoptotic pathway [22,23]. The pathways of apoptosis in the gastric adenocarcinoma cell line SGC-7901 were strongly induced by c9,t11-CLA, which was thought to act as a potential antioxidant and is believed to induce anticarcinogenic activity [24].

Hepatic stellate cells (HSC) are transformed from vitamin A-storing quiescent cells to a myofibroblast (MFB)-like phenotype during hepatic fibrogenesis [25]. Activated HSC plays a crucial role in not only the extracellular matrix (ECM) protein synthesis but also in the appearance of α-smooth muscle actin (α-SMA), which is in response to liver injury caused by hepatotoxin or hepatitis virus [26–28]. Transforming growth factor β (TGFβ) 1 plays a pivotal role in the initiation and perpetuation of fibrogenesis in liver disease by the stimulation of HSC to ECM-producing MFB; the induction of a matrix gene expression including collagens, proteoglycans and structural glycoproteins and the inhibition of matrix degradation by producing inhibitors such as tissue inhibitors of metalloproteinases [29–35].

Many recent studies have shown strong evidence regarding the role of connective tissue growth factor (CTGF) in liver fibrosis as a mediator of TGFβ. The importance of CTGF in HSC biology may be a downstream mediator of the profibrotic and mitogenic actions of TGFβ1, which promotes ECM deposition and fibrogenesis [36]. In cultured human fibroblasts, the CTGF expression was selectively induced by TGFβ but not by any other growth factors [37]. Many researchers have demonstrated that the CTGF stimulates ECM synthesis and fibroblast proliferation like TGFβ [38–41]. In fact, the CTGF may be a better antifibrotic therapeutic target. This is because the long-term inhibition of TGFβ, with various actions, may not be the most appropriate antifibrotic strategy. Williams et al [36] reported that HSC might be an important source of hepatic CTGF in vivo, particularly following stimulation with TGFβ.

The aim of the current study is to evaluate the effect of three isomers of CLA (c9,t11-CLA; t10,c12-CLA and a CLA mixture containing 41% c9,t11 and 44% t10,c12-CLA, respectively) on mitogen-activated protein kinase (MAPK) pathway-dependent apoptosis in HSC and the biological effects of mixed CLA in a diet under a fibrosis model, which is induced by carbon tetrachloride (CCl_4).

2. Materials and methods

2.1. Cell culture and CLA treatment

The rat HSC-T6, an immortalized rat hepatic stellate cell line, which has stable phenotypes and biochemical characters, was kindly provided by Dr. Scott L. Friedman (Mount Sinai School of Medicine, NY, USA). HSC-T6 was cultivated at 37°C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco), penicillin (100 U/ml, Gibco) and streptomycin (100 mg/ml, Gibco). Fully cultivated cells were subcultured in 10-mm dishes by 2×10^5 cells/ml and grown at 37°C in an atmosphere of 5% CO_2 overnight. The medium was replaced with low serum MEM (1% FBS) and supplemented with 180 μM of c9,t11-CLA; t10,c12-CLA and mixed CLA (containing 41% c9,t11 and 44% t10,c12-CLA), respectively, for 24h or 80 μM of them for 48 h in time dependent manner.

2.2. MTS cytotoxicity activity

The survival of the HSC-T6 cells treated with CLA was determined by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, Promega, Madison, WI, USA]. Cells (2×10^4 cells/well) were plated in 96-well microplates, in the presence of CLA (10-200 μM) dissolved in dimethyl sulfoxide for 16 h. MTS/phenazine methosulfate (Cell Titer 96 nonradioactive cell proliferation assay kit, Promega) solution (20:1) was added to the cells at the end of the experiments. The cells were incubated for 1 h at 37°C in a humidified, 5% CO_2 atmosphere, and the absorbance was measured at 490 nm using SUNRISE (TECAN, Salzbrug, Austria).

2.3. DNA fragmentation (4′,6-diamidino-2-phenylindole staining)

In order to determine whether CLA induces apoptosis in HSC-T6 cells, 4′,6-diamidino-2-phenylindole (DAPI, dihydrochloride) (Molecular Probes, Leiden, The Netherlands) staining was performed according to the manufacturer's protocols. Cells (2×10^4 cells/ml) were cultured on to 4-chamber slides and treated with 80 μM of the three isomers in a time-dependent manner until 48 h. After each time point, the cells were washed twice with phosphate-buffered saline (PBS) and fixed by incubation in a 4% paraformaldehyde for 30 min. The PBS-washed cells were incubated in a 1 μg/ml DAPI solution for 30 min in the dark. Following the rinsing of the sample several times in PBS, the slides were mounted. The cells were then observed by a fluorescence microscope (Nicon, Tokyo, Japan).

2.4. Immunoblotting

CLA-treated cells were sonicated in a HEPES-based buffer containing 25 mM of HEPES (pH 7.5), 300 mM of NaCl, 1.5 mM of $MgCl_2$, 0.1% Triton X-100, 0.5 mM of DTT, 20 mM of β-glycerophosphate, 0.1 mM of Na_3VO_4 and Protease Inhibitor Cocktail Tablets (Roche, Mannheim, Germany) at 20% amplitude. The lysate was centrifuged at 3000 rpm for 10 min at 4°C in order to remove cell debris.

Table 1
Nucleotide sequences of primers and PCR conditions in this study

Application	Primers (forward/reverse)	Annealing temperature (°C)
TGFβ1	5′-GCTTTGTACAACAGCACC-3′	60
	5′-CTGCTCCACCTTGGGCTT-3′	
CTGF	5′-CTAAGACCTGTGGAATGGGC-3′	52
	5′-CTCAAAGATGTCATTGCCCCC-3′	
Col1A1	5′-GATGGCTGCACGAGTCACAC-3′	56
	5′-ATTGGGATGGAGGGAGTTTA-3′	
Col1A3	5′-GGCTGCACTAAACACACTGG-3′	50
	5′-TGGAAATAAATAACTGGAAT	
GAPDH	5′-ACCACAGTCCATGCCATCAC-3′	65
	5′-CCACCACCCTGTTGCTGTA-3′	

Table 2
Composition of experimental diets

Ingredients	Control diet (g/100 g diet)	1% CLA diet (g/100 g diet)
Corn starch/sucrose	14·37/46·93	14·37/46·93
Casein	20·00	20·00
L-methionine	0·30	0·30
Cellulose	3·70	3·70
Fat sources		
Lard	5·00	3·95
Corn oil	5·00	5·00
CLA-rich oil[a]		1·05
AIN-76 mineral mix[b]	3·50	3·50
AIN-76 vitamin mix[c]	1·00	1·00
Choline bitartrate	0·20	0·20

[a] Conjugated linoleic acid, linoleic acid 95% containing 41% c9,t11-CLA and 44% t10,c12-CLA (Nu-Chek Prep.).
[b] AIN-76 mineral mix used at this level in 100-mg diet provided the following amounts of each vitamins: thiamine, 0.53 mg; riboflavin, 0.53 mg; pyridoxine HCl, 0.7 mg; niacin, 3.0 mg; calcium pantothenate, 1.6 mg; folic acid, 0.02 mg; vitamin B12, 1.0 mg; vitamin A, 120 R.E.; vitamin E, 3.9 T.E.; vitamin D3, 100 U; menadolin sodium bisulfite, 0.15 mg.
[c] AIN-76 vitamin mix used at this level in 100 mg diet provided the following amounts of each mineral: Ca, 520 mg; P, 400 mg; K, 360 mg; Na, 120 mg; Cl, 157 mg; S, 337 mg; I, 0.02 mg; Fe, 3.5 mg; Mg, 50.6 mg; Zn, 3 mg; Cu, 0.6 mg; Mn, 5.9 mg.

Subsequently, the supernatant was centrifuged at 13,000 rpm for 20 min at 4°C to obtain soluble protein. The protein concentration was determined using the Bradford method [42]. The protein samples (20 μg per lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For immunoblotting, the proteins were electrotransferred to a polyvinylidene fluoride membrane (Schleicher & Schuell, Dassel, Germany). Equal protein loading was confirmed by Coomassie blue staining. After blocking with a 1.5% bovine serum albumin in Tris-buffered saline (TBS), the MAPK and the upstream of the kinase were determined with anti-MAPK 1/2 (Upstate, Lake Placid, NY, USA), anti-phospho-MAPK 1/2 (Upstate), mitogen-activated or extracellular signal-regulated protein kinase (MEK) 1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and MEK2 (Santa Cruz Biotechnology). After washing in TBS, blots were incubated with antirabbit or mouse IgG HRP-conjugated (Promega, Madison, WI, USA). Specific binding was detected using the Super Signal West Dura Extended Duration Substrate (PIERCE, Rockford, IL, USA), and the blots were exposed to Medical X-ray Film (Kodak, Tokyo, Japan).

2.5. RNA extraction and reverse transcriptase-polymerase chain reaction

The expression of the hepatic fibrosis regulated genes was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). The total RNA was extracted from the HSC-T6 cells using RNAzol (TEL-TEST, Friendswood, TX, USA) according to the manufacturer's manual. One microgram of the total RNA was converted to complementary DNA by a reverse transcription reaction and then subsequently applied using a PCR Premix Kit (Bioneer, Daejeon, Korea) in MyCyclerTM (Bio-Rad, Hercules, CA, USA). The primer sets used in the PCR are listed in Table 1.

2.6. Animals and treatment

Five-week-old male Wistar rats ($n=30$) were purchased from Samtako Bio Korea (Kyung Gi-Do, Korea). They were housed in an automatically controlled environment (23±3°C, 50±10% relative humidity) with a 12-h light/dark cycle, an air ventilation frequency of 10 to 20 times per hour, a light intensity of 150–300 lx. The animals were provided with control diet and/or 1% CLA (Nu-Chek Prep., Elysian, MN, USA) diet and water ad libitum. For the quarantine and observation periods, the rats were maintained in a polycarbonate cage [240×390×175 mm (W×L×H)] with a maximum number of five animals per cage. Individual identification cards, showing the group

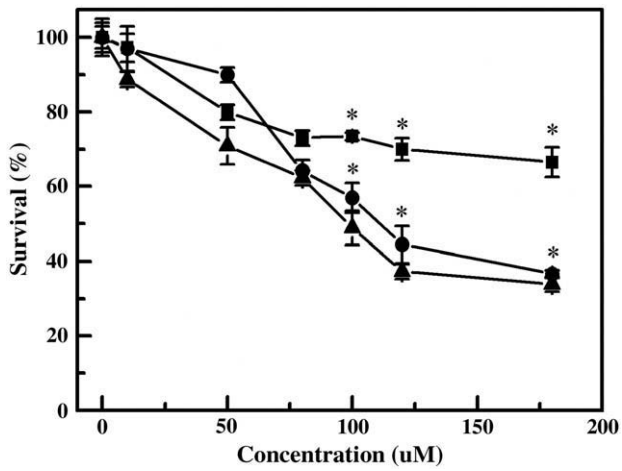

Fig. 1. The isomer specific, dose-dependent effect of the three CLAs on the growth of HSC-T6. Cells were inoculated at 2.0×10^4 cells/well and cultivated in DMEM supplemented with 1% FBS for 24 h with c9,t11-CLA (–●–); t10,c12-CLA (–▲–) and a mixed isomer (c9,t11-CLA:t10,c12-CLA; 41%:44%) (–■–) at 0, 10, 50, 100, 120, 150 and 180 μM. Data are means±S.D. from the three individual experiments. The statistical analysis were performed, each mean value comparing with normal cell with treatment of 80 μM of each CLAs. *$P<.05$.

number and the animal number, were attached to each cage. Each animal was identified by a tail-marking method with an indelible marker. All animals were treated with 10% CCl_4 to induce hepatic fibrosis during all experimental periods. The rats were divided into the following two treatment groups: (1) control diet with tap water ad libitum ($n=15$) and (2) 1% CLA diet with tap water ad libitum ($n=15$). The rats had free access to water, the control diet and 1% CLA diet, which was a powder containing 1.0% CLA (w/w). This diet was a modification of the AIN-76 semipurified diet (Table 2) and was stored at 4°C to prevent oxidation. For analyses, 15 rats were sacrificed at the 2, 4 and 6 weeks, respectively.

2.7. Histopathological analysis

Selected liver samples from each rat were rapidly removed at random and fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin. The samples were cut into sections 3–4 μm. The sections were stained with hematoxylin and eosin (H-E) and with Azan stain special for collagen fibers.

2.8. Immunohistochemistry

The liver sections were deparaffinized in xylene, dehydrated in a graded alcohol series and incubated in a solution of 3% hydrogen peroxide (H_2O_2) in methanol for

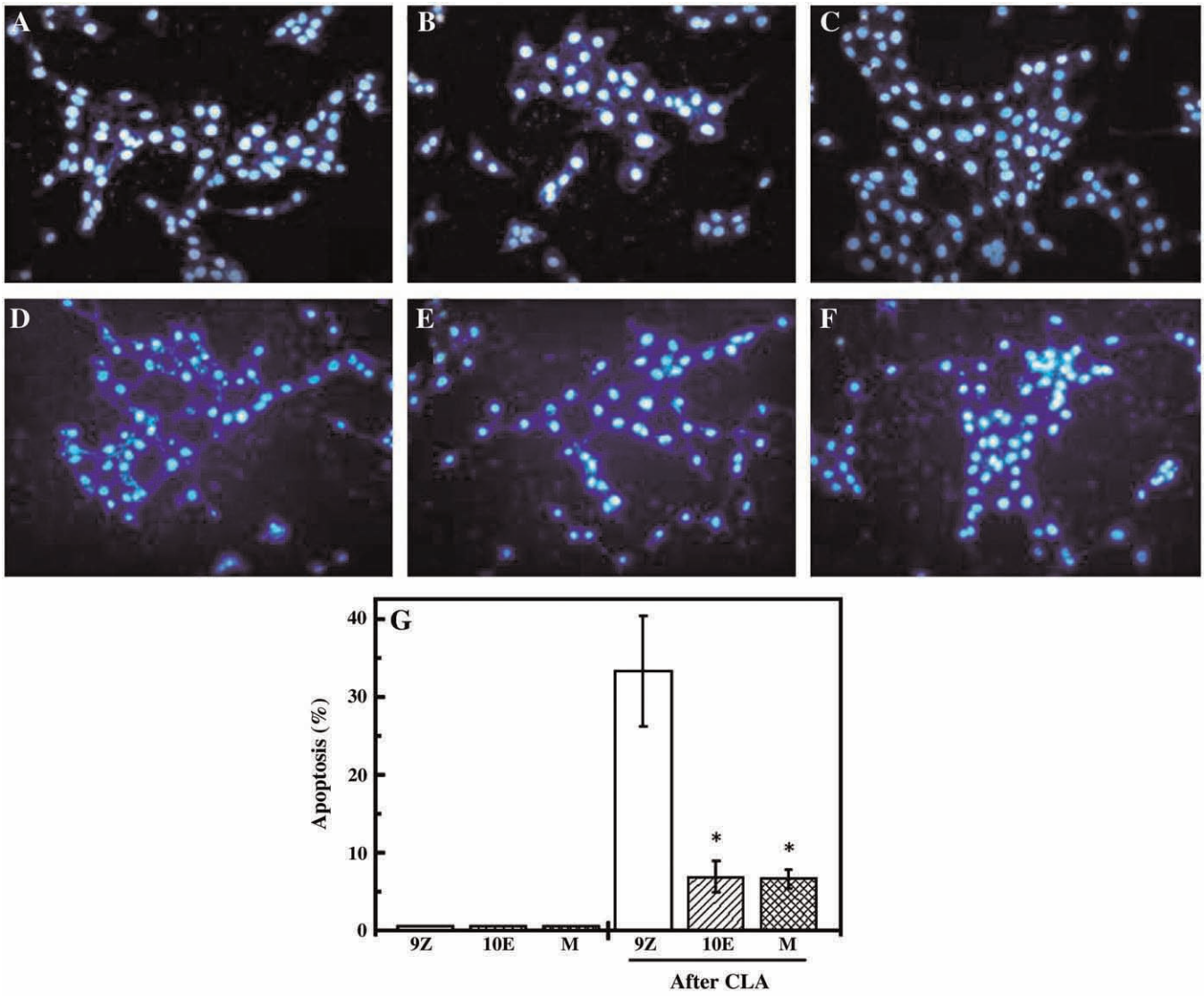

Fig. 2. The evaluation of apoptosis. Nucleus morphology after treatment with (lower panels) and without (upper panels) three CLAs including c9,t11-CLA (9Z; A, D); t10,c12-CLA (10E; B, E) and a mixed isomer (M; c9,t11-CLA:t10,c12-CLA; 41%:44%; C, F) in the HSCs. 2.0×10^4 cells/ml were cultivated on to a slide glass in a 100-mm plastic dish using DMEM supplemented with 10% FBS. The nuclei are visualized by DAPI (4′,6-diamidino-2-phenylindole, dilactate) staining, and they were observed using a fluorescence microscope. Each of the three CLAs was added at 80 μM and the cells were treated for 48 h. Apoptosis was represented by a percentage compared with untreated cells (G). Values are means with their S.D., as shown by vertical bars. The mean values were significantly different from those of the c9,t11 CLA-treated group. *$P<.05$.

10 min. The tissue sections were washed with PBS containing 0.03% nonfat milk and 0.01% Tween 20 and then immunostained with primary antibodies for α-SMA. The antigen-antibody complex was visualized by a labeled streptavidin-biotin method using a Histostatin-plus bulk kit (Zymed Laboratories Inc, South San Francisco, CA, USA). The primary antibody used was: monoclonal anti-α-SMA at a dilution of 1:800 (clone 1A4, Sigma Co, Saint Louis, MO, USA). Nonimmunized goat sera, which were used instead of the primary antibody, served as the negative control.

3. Results

3.1. Apoptosis of HSC by CLA

HSCs were incubated for 24 h with 10–180 μM of c9,t11 (96%); t10,c12 (98%) and a mixed form (c9,t11-CLA:t10,c12-CLA; 41%:44%) CLA. The MTS dye reduction was measured to verify cell viability. Mixed form showed mild decrease of cell viability under 50 μM of treatment. Among the three linoleic acids, c9,t11 and t10,c12-CLA exhibited an intense cytotoxic effect on HSC demonstrating 60% of survival ratio compared to the normal cell by a treatment of 80 μM, while cell survival was slightly affected by the mixed form CLA (Fig. 1).

3.2. The evaluation of DNA fragmentation

In order to determine the apoptotic effect of CLA, the cells were treated for 48 h with 80 μM of three isomers, which represent a 60% reduction of survival. Apoptosis was estimated in vitro by DAPI staining. As shown in Fig. 2, DNA fragmentation was significantly increased in the stellate cells treated with 80 μM of c9,t11-CLA to 38% compared to the normal condition (Fig. 2D and G). There were also moderate changes in DNA fragmentation after treatment with t10,c12 and/or mixed CLA isomers (Fig. 2E–G). These results suggest that 9c,11t-CLA may be the most biologically active isomer regarding the apoptosis of HSC in vitro.

3.3. The effect of CLAs on the expression of cell signaling

When HSCs were exposed to the three isomers, the MEK/MAPK pathway was differently regulated. MEK1 and 2 were not activated under t10,c12-CLA; consequently, extracellular signal-regulated kinase (ERK) could not be activated due to its phosphorylated form, which was increased by the other two CLA isomers, with the c9,t11-CLA isomer being the most potent (Figs. 3 and 4). MEK1 and MEK2 synthese was observed in the mixed isomer and c9,t11-CLA, respectively (Fig. 3). Although t10,c12-CLA did not have an effect on the expression of phosphorylated ERK (p-ERK) through MEK1 and 2, CLA slightly induced apoptosis in HSC (Fig. 4).

3.4. The effect of CLA on the expression of HSC activation markers and collagen mRNA expression

In order to evaluate the effects of CLA on the expression of TGFβ1, CTGF and collagen genes, a quantitative RT-

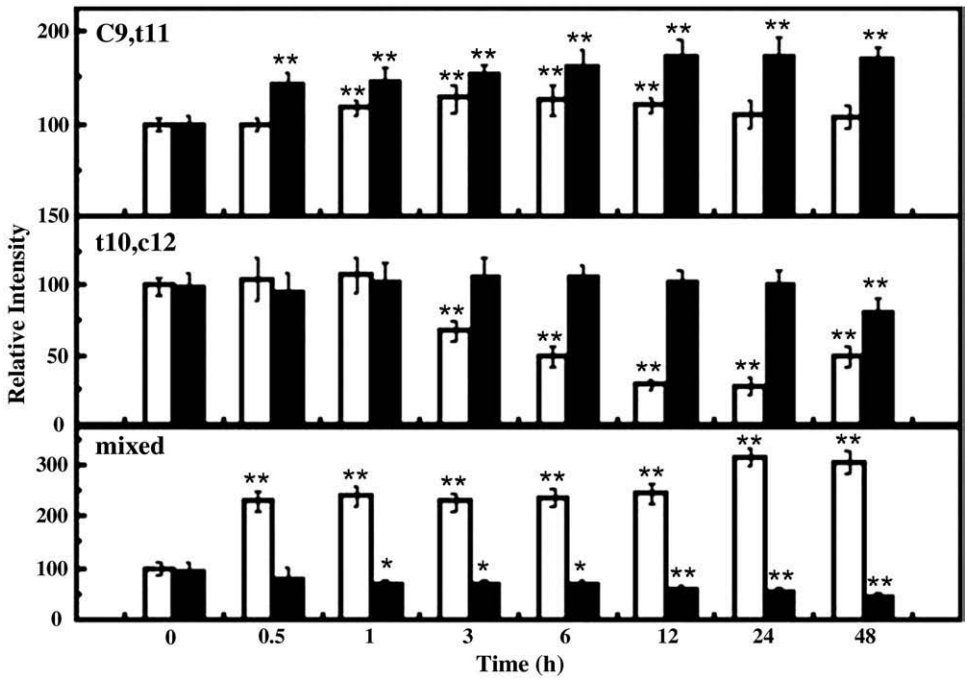

Fig. 3. The effects of CLAs on the expression of MEK/MAPK activation in HSC-T6. HSCs were treated with 80 μM of the three CLAs for indicated time periods. The expression levels of MEK1 and MEK2 were detected by immunoblot detection. The signals obtained from MEK1 and MEK2 were quantified by a Quantity One (ver 4.4.1, Bio-Rad). Open bar, MEK1; hatched bar, MEK2. The values are means with S.D., and they represent a minimum from at least three independent experiments. The mean values were significantly different from those of the nontreated. *$P<.05$; **$P<.01$.

PCR was performed. HSCs were treated with three CLA isomers for 48 h of 80 μM in a time-dependent manner. Although the TGFβ1 expression seemed to be unaffected by CLA for 24 h, the levels significantly reduced after 48 h. CLA down-regulated the expression of TGFβ1 mRNA suppressed CTGF expression as a downstream target of TGFβ1. On the other hand, 80 μM of t10,c12 and mixed-CLA decreased collagen I and collagen III in a time dependent manner, respectively (Fig. 5).

3.5. Histopathology

Dietary CLA significantly inhibited the production of collagen fibers in the livers of rats. Mild fibrosis around the central vein was observed in the control group (Fig. 6A). There was moderate centrilobular necrosis and mild fatty change, however, in the CLA-treated group (Fig. 6D). To determine the extent of the fibrosis, Azan staining was carried out. In the CLA-treated group, collagen fibers were normally present around the central veins (Fig. 6E). In contrast, the number of collagen fibers increased and were deposited along the fibrous septa in the control group (Fig. 6B). Via immunohistochemistry, activated HSCs (myofibroblasts) were identified by α-SMA-positive staining and could be observed only in the central veins, portal veins and hepatic arteries of a normal liver. As liver fibrosis progressed, however, the number of α-SMA-positive cells markedly increased around the portal veins in the control groups at the 2-week period (Fig. 6C). There was less increase in the number of α-SMA-positive cells, however, in the CLA-fed group (Fig. 6F). These

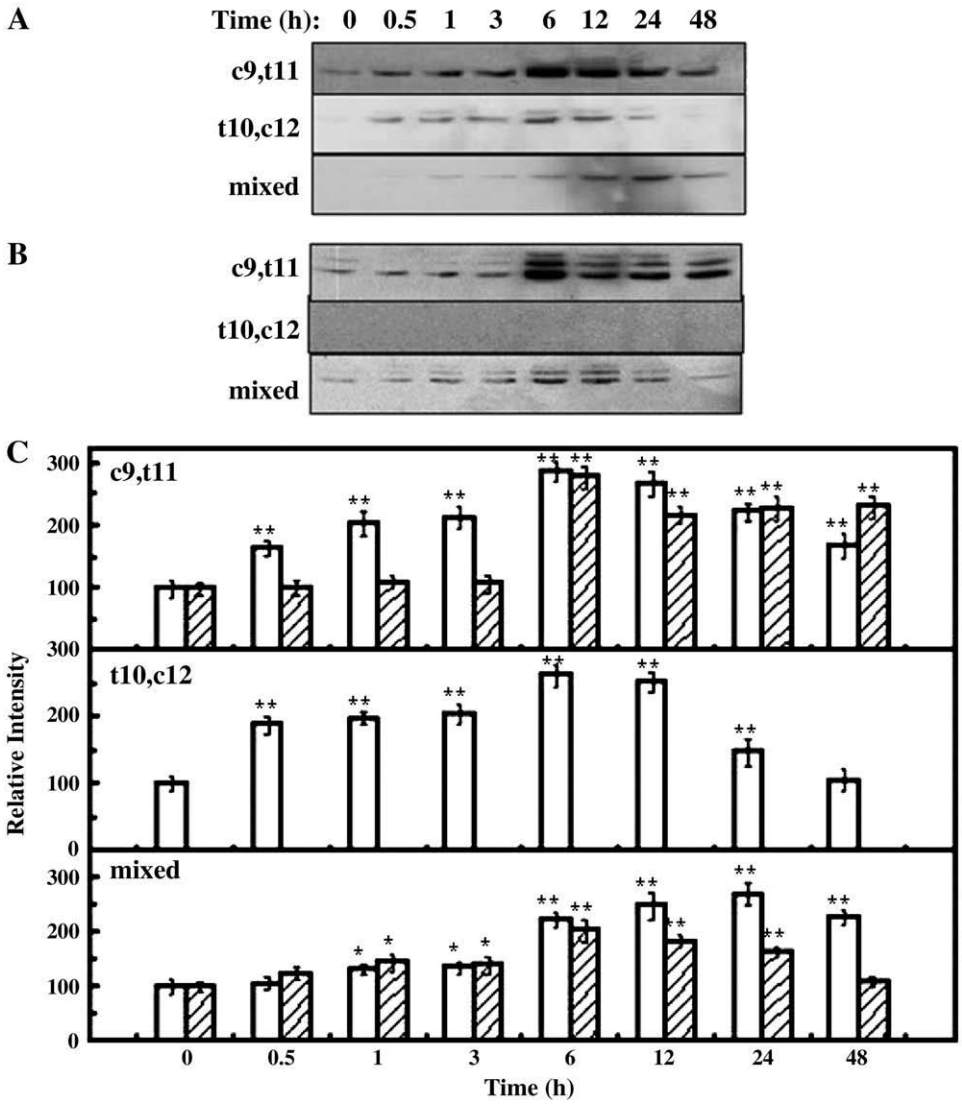

Fig. 4. The effects of CLAs on the total expression of ERK and p-ERK activation in HSC-T6. HSCs were treated with 80 μM of the three CLAs for indicated time periods. The expression levels of the total ERK (A) p-ERK (B) were detected by immunoblot detection. The signals obtained from the total ERK and p-ERK (C) were quantified by a Quantity One (ver 4.4.1, Bio-Rad). Open bar, total ERK; hatched bar, p-ERK. The values are means with standard deviation from at least three independent experiments. The mean values were significantly different from those of the nontreated. *$P<.05$; **$P<.01$.

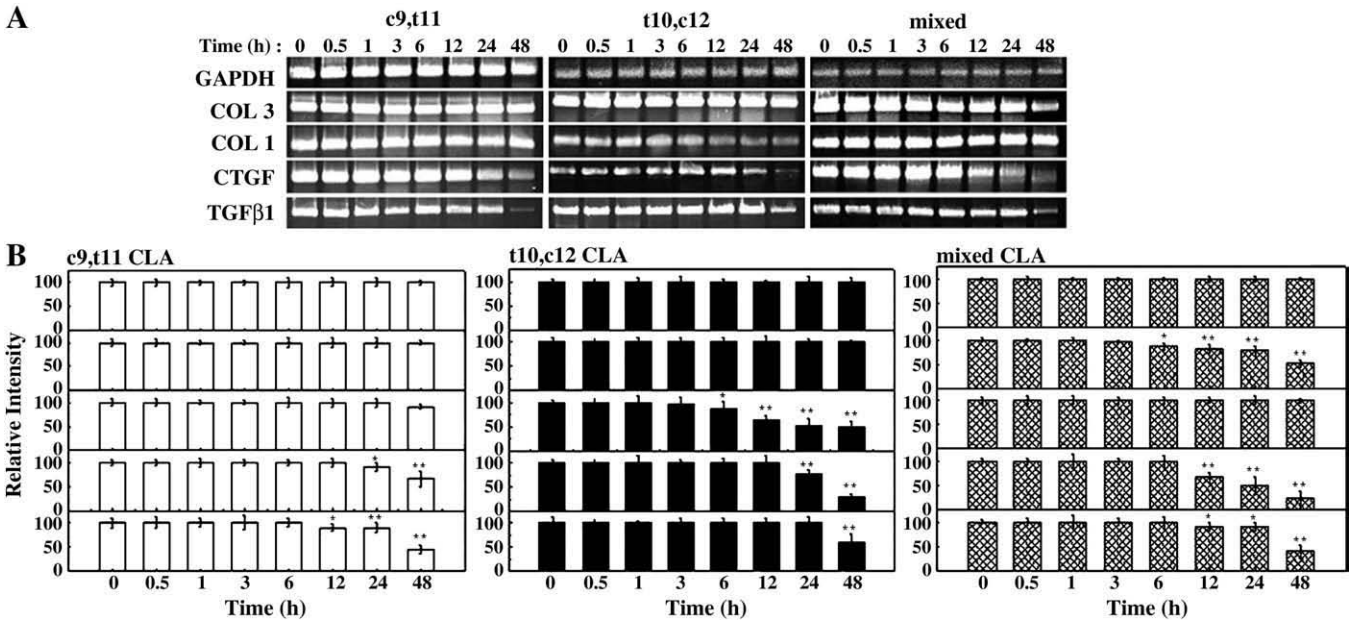

Fig. 5. The effects of CLAs on the expression of an ECM. Total RNA was isolated from HSC-T6, which were treated with three CLA isomers including c9,t11-CLA (left), t10,c12-CLA (middle) and a mixed isomer (c9,t11-CLA:t10,c12-CLA; 41%:44%; right) for 48 h, in a time-dependent manner (A). The expression of the ECM-related genes (Col3A1, Col1A1, CTGF and TGFβ1) was detected by RT-PCR. Amplified DNA products were analyzed on a 15% agarose gel stained with ethidium bromide. The signals obtained were quantified by Quantity One (ver 4.4.1, Bio-Rad), which were normalized to those of the GAPDH and expressed as percentages against the levels of those at the start of the experiment (B). The values are means with S.D. from at least three independent experiments. The mean values were significantly different from those of the nontreated. $*P<.05$; $**P<.01$.

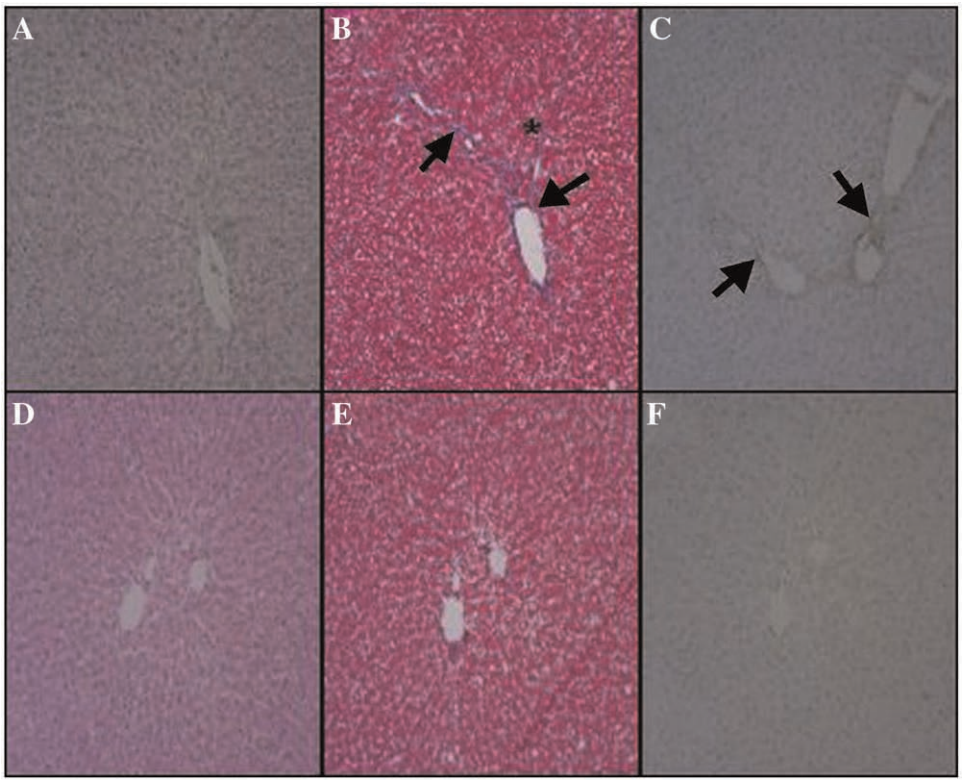

Fig. 6. Dietary CLA inhibits the production of collagen fiber in rat liver. The figures shows H-E- and Azan-stained paraffin sections of a rat liver ($n=5$/group). The rats were fed the control diet (panel A, B) and 1% CLA supplemented diet (panel D, E) for 2 weeks. α-SMA is also detected by immunohistochemistry in the control (C) group, which was fed the CLA supplemented diet (F). In the control group (A–C), fibrous septa (asterisk), expanding from the central vein, can be observed and the number of collagen fibers (arrow) increased and were deposited along the fibrous septa. The number of myofibroblasts (arrow head) increased around the central veins. In the CLA-treated group (D–F), moderate centrilobular necrosis, mild fatty change and collagen fibers are present around central veins. There is no increase or activation of the myofibroblasts in the CLA treated group (original magnification ×33).

results indicate the hepatoprotective effects of CLA in CCl_4-treated rat liver fibrosis.

4. Discussion

In this article, we report on the apoptotic effects of three commercial CLA and their isomers, c9,t11-CLA; t10,c12-CLA and a CLA mixture containing 41% c9,t11 and 44% t10,c12-CLA. The effects were determined using HSC in vitro. Moreover, the rats were fed diets containing 1% CLA had enhanced biological efficiency and a reduced amount of collagen fiber and an increase in the protective effect against liver damage in activated HSCs.

The c9,t11-CLA isomer exhibited the greatest potency against cell apoptosis, and the t10,c12-CLA and mixed isomers were moderately effective. The c9,t11-CLA isomer revealed significant induction of MEK1 and MEK2 (Fig. 3) suggesting MEK/MAPK pathway-dependent apoptosis of HSCs (Fig. 1). It is interesting to note that t10,c12-CLA-stimulated apoptosis of HSCs, at similar levels, were comparable with the mixed isomers, without affecting the phosphorylation of ERK (Figs. 2 and 4). These results indicate that signals activated by t10,c12-CLA might be different from the other two CLAs, although they lead to the same results as apoptosis. On the other hand, the expression of both α1 collagen types I (Col1A1) and III (Col3A1) was inhibited parallel with the expression of TGFβ1 and CTGF, a downstream target of TGFβ1, under CLA treatment. Notably, the Col3A1 mRNA expression was significantly inhibited in the mixed CLA-treated HSCs, indicating that CTGF might be a key factor in reducing the expression of Col3A1, thus leading to ECM decomposition.

During the hepatic damage, HSCs transform into myofibroblasts in response to the platelet-derived growth factor-BB and insulin-like growth factor-1, accompanied by increments of a HSC activation marker such as α-smooth muscle actin, thus resulting in ECM accumulation [43,44]. ECM metabolisms are highly regulated by TGFβ1 functions, which modulate the synthesis of ECM components by activating the MAPK pathway and the proliferation of activated HSCs, which respond to receptors for individual growth factors [44].

A cell's fate contributes to the homeostatic balance of cell proliferation and apoptotic cell death. One path of recovery from liver fibrosis is to reduce activated HSC in the liver by the elimination of MFB by programmed cell death [12,45,46]. A preliminary report suggests that apoptosis can regulate the number of activated stellate cells during liver injury [47]. Saile et al. [48] reported that by using CD95-activating antibodies, the transition of more than 95% cells into apoptosis was evident at each activation step. These data suggest that apoptosis might represent an important mechanism in terminating the proliferation of activated HSCs.

The MAPK pathway plays a crucial role in the cellular response to environmental stress and inflammation, which can lead to gene expression, proliferation and apoptosis in HSC. Fibronectin (FN) and inflammatory cytokines increase MAPK activity, stimulate AP-1 activity and increase transgenic expression, suggesting that the MAPK family may play an important role in the regulation of the matrix metalloproteinase expression by cytokines and FN in HSCs [49]. MAPK kinases such as MEK1 and 2 phosphorylate ERK result in the extracellular signal transduction to the nucleus by stimulating downstream elements including c-*myc*, ELK and Est. Control of HSC apoptosis, however, by MEK and ERK activation has not been fully established yet.

In recent studies, CLA has been reported to induce apoptosis of not only cancer cells, but also normal rat mammary epithelial cells [13]. In addition, CLA can regulate cell proliferation, the number of reduce cells in preadipocyte cultures [50]. The effect of CLA on hepatic stellate cells has not yet been elucidated yet. In the current study, we have demonstrated that differently regulated-apoptosis in HSCs attributed to the physiologic levels of the three isomers in the stellate cells, which depend on the activation of the MEK/MAPK pathway. In addition, in terms of the apoptosis of activated HSC, our results suggest that CLA may provide a novel therapeutic effect as an antifibrotic agent.

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