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RESEARCH ARTICLES

Influence of conjugated linoleic acid isomers on the metastasis of colon cancer cells in vitro and in vivo ☆

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Abstract

This study investigated the isomer-specific effects of *cis*-9,*trans*-11 (*c*9,*t*11) and *trans*-10,*cis*-12 (*t*10,*c*12) conjugated linoleic acid (CLA) on the metastasis of colon cancer cells in vitro and in vivo. Cell migration was examined by a Boyden chamber assay in SW480 cells. MMP-9 activity was monitored by gelatin zymography, and MMP-9 protein and mRNA levels were determined by Western blot and RT-PCR analysis, respectively, in SW480 cells. For the experimental metastasis, BALB/c mice were injected intravenously with CT-26 cells in the tail vein. Mice were fed a diet containing either no CLA or 0.1% *c*9,*t*11 or *t*10,*c*12 CLA for 4 weeks. In experimental metastasis, the numbers of pulmonary nodules were significantly lower in mice fed CLA isomers than in mice fed a control diet ($P<.05$). Results from the Boyden chamber assay revealed that *c*9,*t*11 CLA significantly inhibited cell migration ($P<.05$), whereas *t*10,*c*12 CLA had no effect on cell migration. The activity of MMP-9 was significantly inhibited by *c*9,*t*11 CLA ($P<.05$) but not by *t*10,*c*12 CLA. However, neither MMP-9 protein nor mRNA levels were altered by either of these CLA isomers. We have demonstrated that diets containing 0.1% *c*9,*t*11 and *t*10,*c*12 CLA were equally effective in inhibiting colon cancer cell metastasis in vivo. However, in vitro, only *c*9,*t*11 but not *t*10,*c*12 inhibited colon cancer cell migration and MMP-9 activity.

Keywords: Conjugated linoleic acid isomer; Migration; Matrix metalloproteinase; Metastasis; Colon cancer

1. Introduction

Colon cancer is one of the leading causes of deaths in Western countries and the incidence of this disease is rapidly increasing in Korea [1]. Death from colon cancer is often the result of systemic metastasis [2]. Thus, metastasis is a major issue in successful colon cancer treatment.

The process of metastasis consists of a series of tumor–host interactions that involve cell migration and the breakdown of the basement membrane by extracellular matrix (ECM)-degrading enzymes, which include serine proteinases, cysteine proteinases and matrix metalloproteinases (MMPs) [3]. Among these enzymes, MMPs, which are zinc-dependent endopeptidases, represent a large family that plays a key role in angiogenesis, tumor invasion and metastasis [4–6]. MMP activity is regulated in at least three levels: transcription, proteolytic activation of the zymogen form and inhibition of the active enzyme [7]. A large variety of external stimuli, such as cytokines, growth factors and cell–cell interactions, can stimulate the expression of various MMPs [8]. Because MMPs have many physiological functions in metastasis, inhibition of the activity of MMPs by dietary factors holds great promise for the prevention or inhibition of metastasis.

Diet, especially fat intake, has been considered to be the most important influence on colon cancer development and progression [9]. In this connection, it is noteworthy that conjugated linoleic acid (CLA), a mixture of positional and geometric isomers of linoleic acid found mainly in dairy and meats such as beef and lamb [10], has been widely shown to possess anticarcinogenic activity against various cancers in both in vitro and in vivo models [11–14]. CLA isomer mixtures are usually synthesized by alkali isomerization of linoleic (*cis*-9,*cis*-12 LA) acid. The *cis*-9,*trans*-11 (*c*9,*t*11) and *trans*-10,*cis*-12 (*t*10,*c*12) CLA isomers represent about

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80–90% of total CLA isomers, with *cis*-9,*cis*-11, *trans*-9,*cis*-11, *trans*-9,*trans*-11, *cis*-10,*cis*-12, *cis*-10,*trans*-12 and *trans*-10,*trans*-12 CLA present in minor concentrations [15].

The roles of specific isomers in various biological activities observed with commercial supplements of CLA have been reviewed by Pariza et al. [16]. The *t*10,*c*12 CLA isomer has been reported to exhibit the greatest potency against HT-29 [17] and Caco-2 colon cancer cell growth [18]. Previously, we reported that CLA mixture inhibited the metastatic properties of SW480 colon cancer cells [19]. Visonneau et al. [20] also showed that 1% dietary CLA mixture decreased growth and pulmonary metastasis in severe combined immunodeficient mice transplanted with MDA-MB 468 human breast cancer cells. However, there are few reports assessing the effects of individual CLA isomers on the process of metastasis. In this study, we examined the effects of specific CLA isomers, *c*9,*t*11 and *t*10,*c*12, on the process of metastasis of colon cancer both in vitro and in an animal model.

2. Materials and methods

2.1. Materials and reagents

Dulbecco's modified Eagle's medium/F12 (DMEM/F12) was obtained from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from JRH (Lenexa, KS, USA). CLA isomers were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). CLA isomers for cell culture studies were complexed to fatty acid-free albumin (1 mmol/L BSA/4 mmol/L fatty acid) and kept at −20°C. Antibody for MMP-9 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other reagents were from Sigma (St. Louis, MO, USA).

2.2. Cell culture

SW480 human colon cancer cells and CT-26 mouse colon cancer cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in DMEM/F12, containing 10% FBS, 100,000 U/L penicillin and 100 mg/L streptomycin. The medium was replaced every 2–3 days.

2.3. Boyden chamber assay

Colon cancer cell migration was assayed using a microchemotaxis chamber (Neuro Probe, Inc., Gaithersburg, MD, USA) and PVPF filters (Osmonics, Inc., Minnetonka, MN, USA) with 8-μm-diameter pores [21]. SW480 cells (1.1×10^5 cells/well) suspended in SFM containing 0, 1, 2 or 4 μmol/L CLA isomers with 100 μg/L PMA were carefully transferred to the upper chambers. The lower chambers were filled with 10% FBS medium to attract cells. The chamber was placed in a humidified incubator at 37°C under 5% CO_2 atmosphere for 16 h. Following gentle removal of a filter from the chamber, the upper side was wiped with a paper to remove the cells on that side, and colon cancer cells that had migrated to the lower side of the filter were fixed in 100% methanol and stained with Diff-Quick stain solution (Dade Behring, Newark, DE, USA). The cells on each filter were counted in five randomly selected microscopic fields (×400). Migration was determined as the mean number of cells that had migrated and was expressed as a percentage of control (−PMA, 0 μmol/L CLA). All experiments were performed independently three times.

2.4. Gelatin zymography assay

MMP-9 activity in the culture medium was monitored by gelatin zymography [22]. SW480 cells were plated into six-well culture plates at a dose of 1×10^6 cells/well and cultured in medium containing 10% FBS to form nearly confluent cell monolayers. Cell monolayers were treated with various concentrations of CLA isomers and PMA (100 μg/L). After 48 h of incubation, the conditioned medium was collected and then concentrated 10-fold in Centricon (Millipore, Bedford, MA, USA), and 50 μl of concentrated medium was electrophoresed on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) containing 1 g/L gelatin. The gel was incubated twice for 30 min at room temperature in renaturing buffer (2.5% Triton X-100) and washed once for 30 min at room temperature in developing buffer (50 mmol/L Tris base, 50 mmol/L Tris-HCl, 0.2 mol/L NaCl, 5 mmol/L $CaCl_2$, 0.02% Brij 35), and then it was incubated for a further 16 h at 37°C in fresh developing buffer. The gel was stained with 0.5% Coomasssie Brillant Blue R-250 for 30 min and destained in destaining solution (methanol/acetic acid/water=50:10:40). Gelatinolytic activity appeared as a clear band on a blue background.

2.5. Western blot analysis

MMP protein levels in the culture medium were determined by Western blotting. The conditioned media were collected and concentrated by the same procedures as for the gelatin zymography. Proteins were separated by 4–20% gradient SDS-PAGE and transferred to an immobilon-P transfer membrane (Millipore, Bedford, MA, USA). The membrane was blocked in 5% milk/TBST (20 mmol/L Tris-HCl, 137 mmol/L NaCl, 0.1% Tween 20, pH 7.4) for 1 h at room temperature, incubated with monoclonal anti-MMP-9 antibody/5% milk-TBST (1:1000 dilution) for 1 h at room temperature and washed for 30 min at room temperature in TBST. The membrane was then incubated with antimouse IgG horseradish peroxidase/5% milk-TBST (1:10,000 dilution, Amersham, Buckinghamshire, England). The presence of bound antibody was detected by chemiluminescence using Supersignal West Dura Extended Duration Substrate (Pierce, Illinois, USA), and the intensity of the immunoreactive bands was quantified using a densitometer (Sanyo, Japan).

2.6. RNA extraction and reverse transcription polymerase chain reaction

Total cellular RNA was extracted from SW480 cells using the TRI reagent (Sigma). The purity and integrity of

Table 1
Compositions of experimental diets (g/kg)

Ingredients	Control	*C*9,*t*11 CLA	*t*10,*c*12 CLA
Cornstarch	529.4859	529.4860	529.4860
Casein	200.0	200.0	200.0
Sucrose	100.0	100.0	100.0
Soybean oil	70.0	68.89	68.89
CLA isomer[a]			
*c*9,*t*11 CLA		1.11	
*t*10,*c*12 CLA			1.11
Fiber (cellulose)	50.0	50.0	50.0
Mineral mix[b]	35.0	35.0	35.0
Vitamin mix[c]	10.0	10.0	10.0
L-Cysteine	3.0	3.0	3.0
Choline	2.5	2.5	2.5
Ronoxan A[d]	0.0001		
tert-Butylhydroquinone	0.014	0.014	0.014

[a] CLA isomers had a purity of 90%.

[b] AIN mineral mixture 93G contains the following compounds (per kilogram): calcium carbonate, anhydrous, 357 g; potassium phosphate, monobasic, 196 g; potassium citrate, tri-potassium, monohydrate, 70.78 g; sodium chloride, 74.00 g; potassium sulfate, 46.60 g; magnesium oxide, 24.00 g; ferric citrate, 6.06 g; zinc carbonate, 1.65 g; manganous carbonate, 0.63 g; cupric carbonate, 0.30 g; potassium iodate, 0.01 g; sodium selenate, anhydrous, 0.01025 g; ammonium paramolybdate, hydrate, 0.00795 g; sodium meta-silicate, hydrate, 1.45 g; chromium potassium sulfate, 0.275 g; lithium chloride, 0.0174 g; boric acid, 0.0815 g; sodium fluoride, 0.0635 g; nickel carbonate, 0.0318 g; ammonium vanadate, 0.0066 g; powdered sucrose, 221.026 g.

[c] AIN vitamin mixture 93G contains the following compounds (per kilogram): nicotinic acid, 3.000 g; Ca-pantothenate, 1.600 g; pyridoxine-HCl, 0.700 g; thiamin-HCl, 0.600 g; riboflavin, 0.600 g; folic acid, 0.200 g; D-biotin, 0.020 g; vitamin B_{12} (cyanocobalamin) (0.1% in mannitol), 2.500 g; vitamin E (all-rac-α-tocopheryl acetate) (500 IU/g), 15.000 g; vitamin A (all-trans-retinyl palmitate) (500.0 IU/g), 0.800 g; vitamin D_3 (cholecalciferol) (400 IU/g), 0.250 g; vitamin K (phylloquinone), 0.075 g; powdered sucrose, 974.655 g.

[d] Antioxidant contained in the CLA isomer solution.

the RNA samples were assessed by $A_{260/280}$ spectrophotometric measurement. After measurement of the concentration, 3 μg total RNA was reverse transcribed into cDNA by reverse transcriptase (Invitrogen, California, USA), and amplification of cDNA was performed as previously described [23]. The sequences of the primers were as follows: MMP-9, sense 5′ -CAT CGT CAT CCA GTT TGG TG-3′ and antisense 5′ -GGT GTA GAG TCT CTC GCT GG-3′ ; β-actin, sense 5′ -GTT TGA GAC CTT CAA CAC CCC-3′ and antisense 5′ -GTG GCC ATC TCC GCT CGA AGT-3′ . Polymerase chain reaction (PCR) conditions for MMP-9 and β-actin were as follows: denaturation at 94°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 1 min. For each combination of primers, the kinetics of the PCR amplification were examined and the number of cycles corresponding to the plateau determined. The optimal numbers of PCR cycles of MMP-9 and β-actin within exponential ranges were 32 and 35, respectively. PCR products were resolved on 1% agarose gels, which were then stained with ethidium bromide before being photographed.

2.7. Experimental metastasis

2.7.1. Animals and diets

Four-week-old male BALB/c mice were obtained from Daehan Biolink (Shungbuk, Korea) and were initially fed a stock diet (Harlan Teklad Laboratory Animal Diets, Wisconsin, USA) for a 1-week adjustment period. Mice were randomly divided into three groups. The experimental diets were based on AIN-93G diet (Table 1) containing CLA isomers at 0 or 0.1% (w/w) [24], and soybean oil was reduced by the amount of CLA isomers added such that the total amount of fat equaled 7% (w/w). The animals were maintained according to the animal care guidelines of the Dankook University ethics committee. They were housed in a temperature-controlled room (at 23±2°C) with light from 0600 to 1800 under specific pathogen-free

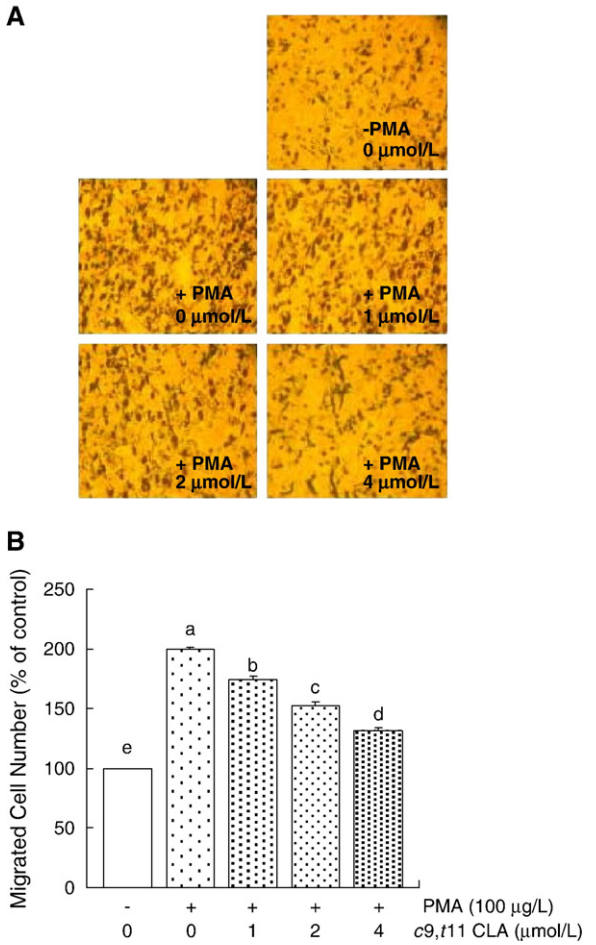

Fig. 1 . Effects of the *c*9,*t*11 CLA isomer on PMA-induced migration in SW480 cells. The cells were cultured in serum-free medium with or without 100 μg/L PMA in the absence or presence of various concentrations (1, 2 or 4 μmol/L) of *c*9,*t*11 CLA for 16 h in a Boyden chamber. The microphotographs of filters (A) and quantitative analysis of the Boyden chamber migration assay (B) are shown. The number of migrated cells was expressed as a percentage of control (−PMA, 0 μmol/L *c*9,*t*11 CLA). Each bar represents the mean±S.D. calculated from three independent experiments. Means without a common letter differ, $P<.05$.

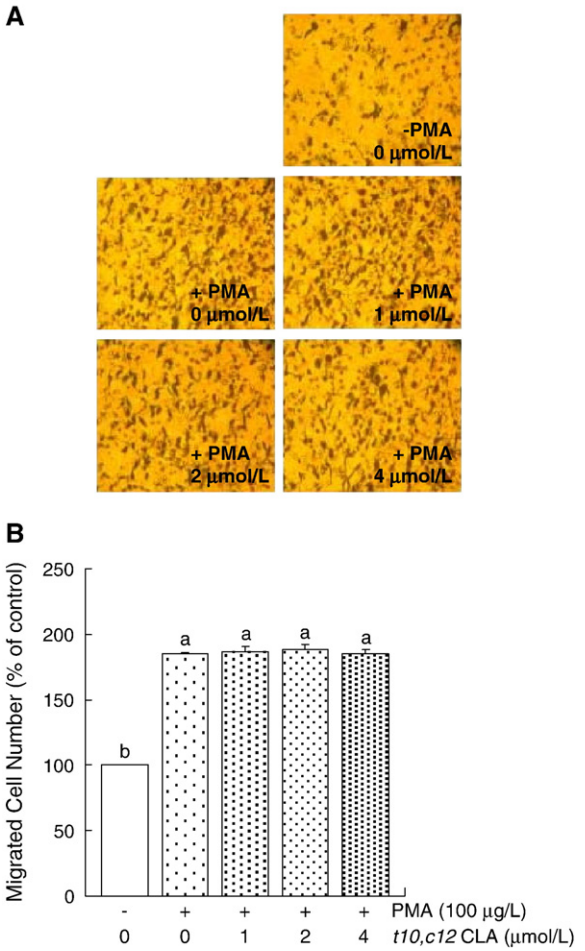

Fig. 2. Effects of the *t*10,*c*12 CLA isomer on PMA-induced migration in SW480 cells. The cells were cultured in serum-free medium with or without 100 μg/L PMA in the absence or presence of various concentrations (1, 2 or 4 μmol/L) of *t*10,*c*12 CLA for 16 h in a Boyden chamber. The microphotographs of filters (A) and quantitative analysis of the Boyden chamber migration assay (B) are shown. The number of migrated cells was expressed as a percentage of control (−PMA, 0 μmol/L *t*10,*c*12 CLA). Each bar represents the mean±S.D. calculated from three independent experiments. Means without a common letter differ, $P<.05$.

conditions. The humidity was automatically maintained at 50±10%.

2.7.2. Lung metastasis

Mice were injected intravenously in the tail with CT-26 colon cancer cells suspended in SFM at a dose of 5×10^4 cells/mouse. Mice were fed the experimental diets for 4 weeks, and they were sacrificed and their lungs were removed. Lungs were stained with Bouin's solution and the numbers of metastatic colonies were counted.

2.8. Statistical analysis

Statistical analysis was performed using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Data were expressed as means with standard deviations and analyzed via ANOVA. Statistically significant differences between the means of the groups were tested at $\alpha=0.05$ using Duncan's multiple range test.

3. Results

3.1. c9,t11 CLA inhibits migration of SW480 cells

Cell migration plays an important role in the metastasis process. Because PMA, a powerful tumor promoter, has been shown to induce tumor cell invasion/migration [25,26] and increase MMP activity [27,28], we examined whether the individual CLA isomers are able to prevent PMA-induced migration of SW480 cells in a Boyden chamber assay. PMA induced the migration of SW480 cells toward the medium containing 10% FBS used as the chemoattractant, and the *c*9,*t*11 CLA isomer significantly

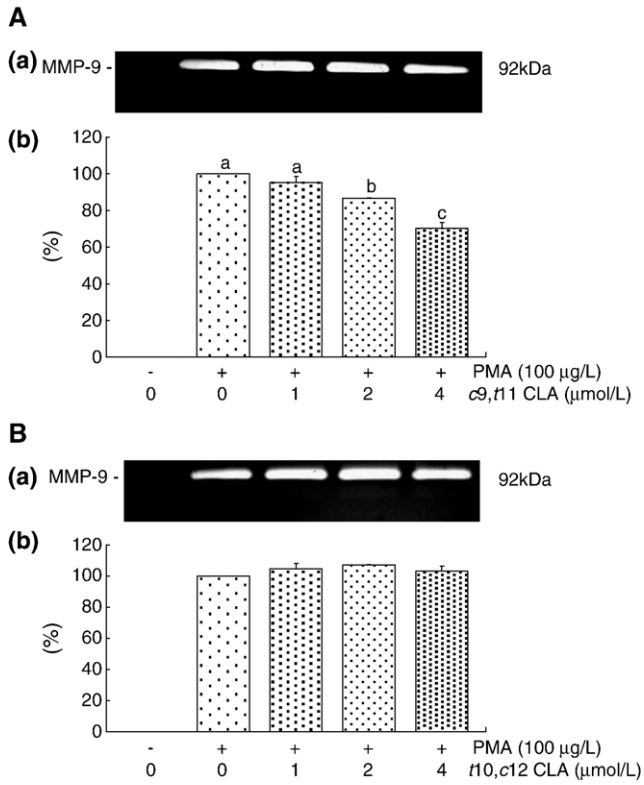

Fig. 3. Effects of CLA isomers on PMA-induced MMP-9 activity in SW480 human colon cancer cells. Cells were plated in six-well plates at a density of 1×10^6 cells/well in DMEM/F12 supplemented with 10% FBS. The monolayers were incubated in serum-free medium with or without 100 μg/L PMA in the absence or presence of various concentrations (1, 2 or 4 μmol/L) of *c*9,*t*11 CLA (A) or t10,c12 CLA (B) for 48 h. Medium was collected and concentrated for zymography. (a) Photographs of the MMP-9 bands, which are representative of three independent experiments, are shown. (b) Quantitative analysis of the bands. The relative abundance of each band was quantified, with the control (100 μg/L PMA, 0 μmol/L CLA) level set at 100%. Each bar represents the mean±S.D. calculated from three independent experiments. Comparisons among groups that yielded statistically significant differences ($P<.05$) are indicated by different letters above each bar.

inhibited the PMA-induced cell migration ($P<.05$) (Fig. 1). However, the *t*10,*c*12 CLA isomer had no effect on the cell migration (Fig. 2).

3.2. c9,t11 CLA isomer decreases MMP-9 activity in SW480 cells

To examine whether CLA isomers decrease the activity of MMP-9, which is involved in ECM breakdown, cells were treated with various concentrations of CLA isomers in the absence or presence of 100 μg/L PMA and 12-h conditioned media were collected for gelatin zymography. Clearance of the gelatin substrate by gelatinases with M_r of 92,000 (MMP-9) was detected. The activity of MMP-9 induced by PMA in SW480 human colon cancer cells was significantly inhibited by the *c*9,*t*11 CLA isomer ($P<.05$), but not by the *t*10,*c*12 CLA isomer (Fig. 3).

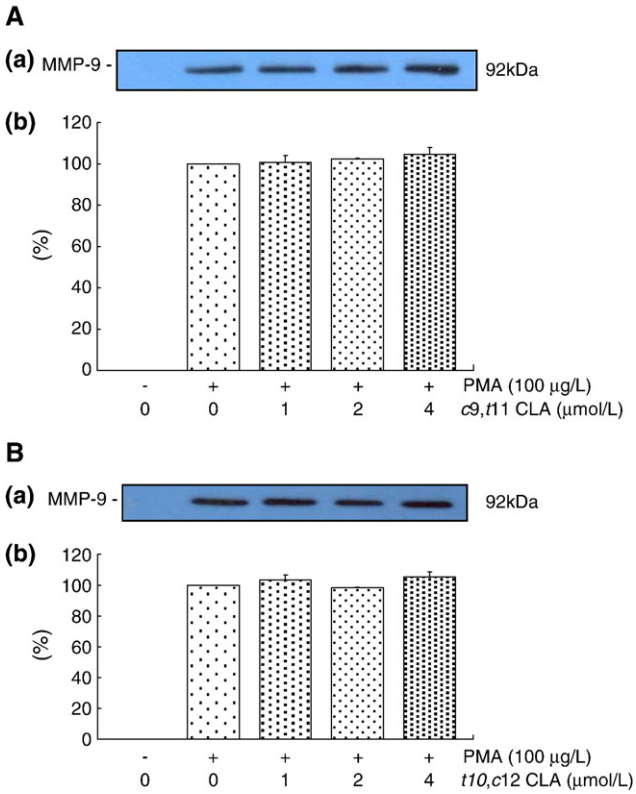

Fig. 4. Effects of CLA isomers on PMA-induced MMP-9 protein levels in SW480 human colon cancer cells. Cells were plated in six-well plates at a density of 1×10^6 cells/well in DMEM/F12 supplemented with 10% FBS. The monolayers were incubated in serum-free medium with or without 100 μg/L PMA in the absence or presence of various concentrations (1, 2 or 4 μmol/L) of *c*9,*t*11 CLA (A) or *t*10,*c*12 CLA (B) for 48 h. Medium was collected and concentrated for Western blotting. (a) Photographs of the MMP-9 bands, which are representative of three independent experiments, are shown. (b) Quantitative analysis of the bands. The relative abundance of each band was quantified, with the control (100 μg/L PMA, 0 μmol/L CLA) level set at 100%. Each bar represents the mean±S.D. calculated from three independent experiments.

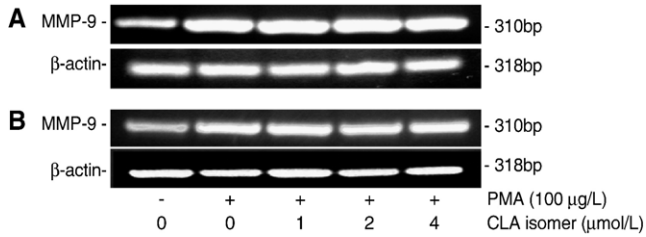

Fig. 5. Effects of CLA isomers on PMA-induced MMP-9 mRNA levels in SW480 human colon cancer cells. The cells were cultured in serum-free medium with or without 100 μg/L PMA in the absence or presence of various concentrations (1, 2 or 4 mol/L) of *c*9,*t*11 CLA (A) or *t*10,*c*12 CLA (B) for 12 h. Total RNA was isolated and RT-PCR was performed. Photographs of the ethidium bromide-stained gels, which are representative of three independent experiments, are shown.

3.3. Neither c9,t11 nor t10,c12 alters MMP-9 protein and mRNA expression in SW480 cells

The protein levels of MMP-9 were assayed by Western blot analysis. MMP-9 protein expression in SW480 human colon cancer cells was induced by PMA, which was not changed by either of the CLA isomers (Fig. 4). We performed RT-PCR analysis to determine the mRNA expression of MMP-9. As shown in Fig. 5, PMA significantly increased MMP-9 mRNA levels, but neither *c*9,*t*11 nor *t*10,*c*12 influenced PMA-induced MMP-9 mRNA expression in SW480 cells.

3.4. Both c9,t11 and t10,c12 decrease experimental metastasis in mice

To examine the effect of CLA isomers on experimental metastasis, mice were injected intravenously in the tail vein with CT-26 colon cancer cells and were fed the experimental

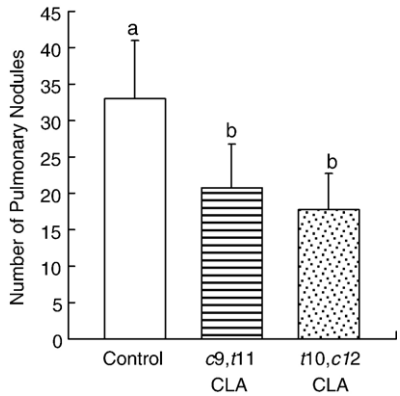

Fig. 6. Effects of CLA isomers on experimental metastasis in BALB/c mice. Four-week-old male BALB/c mice were injected intravenously with CT-26 cells in the tail vein at a dose of 5×10^4 cells/mouse. Mice were fed a diet containing no CLA or 0.1% of either *c*9,*t*11 or *t*10,*c*12 CLA (w/w) for 4 weeks. Mice were then sacrificed and their lungs were removed. The lungs were stained with Bouin's solution and the numbers of metastatic colonies were counted. Each bar represents the mean±S.D. Comparison among groups that yielded statistically significant differences ($P<.05$) are indicated by different letters above each bar.

diet containing either 0.1% *c*9,*t*11 or 0.1% *t*10,*c*12 for 4 weeks. Lungs of mice fed the CLA isomer diets had significantly fewer pulmonary nodules than those of mice fed the control diet ($P<.05$) (Fig. 6). There was no difference in the number of pulmonary nodules between the groups fed *c*9,*t*11 and *t*10,*c*12 CLA. We did not quantify the sizes of the lung nodules because they did not appear to be affected by experimental diets.

4. Discussion

Because most deaths from cancer are related to metastasis [2], studies to determine the antimetastatic effect of various dietary components on colon cancer are very meaningful. Metastasis is a multiple-stage process consisting of cell adhesion to the ECM, degradation of ECM, and cancer cell motility followed by cell detachment [29]. Many studies have shown that CLA has chemopreventive effects in various cancers, including colon cancer [11–14]. We previously reported that a CLA isomer mixture reduced the PMA-induced migration of SW480 cells [19]. However, the effects of the individual CLA isomers on metastasis have not been determined. Therefore, in this study, we investigated whether the predominant CLA isomers present in commercial CLA preparations, *c*9,*t*11 and *t*10,*c*12, can alter cell migration and the activity, protein levels and mRNA levels of MMP-9, which is a key enzyme involved in the degradation of ECM. We also examined whether CLA isomers have any effect on experimental metastasis in vivo.

Cell migration is a crucial step in metastasis. In this study, cell migration induced by PMA was significantly inhibited by the *c*9,*t*11 CLA isomer ($P<.05$), as assayed by the Boyden chamber assay (Fig. 1). Other investigators have shown that *t*10,*c*12 CLA is more potent and efficacious than *c*9, *t*11 CLA in inhibiting proliferation of HT-29 [30] and Caco-2 colon cancer cells [18]. However, the present results showed that *c*9,*t*11 CLA was more effective than *t*10,*c*12 CLA in inhibiting cell migration. Yang et al. [31] also found that the *c*9,*t*11 CLA isomer could inhibit the invasion of the reconstituted basement membrane by SGC-7901 gastric carcinoma cells.

MMPs are important proteolytic enzymes during organ development and tissue regeneration, but they also play important roles in cancer invasion and metastasis [32]. Particularly, MMP-2 and MMP-9 play important roles in tumor invasion and angiogenesis, and, therefore, tumor metastasis can be inhibited by blocking MMP synthesis and activity. Harris et al. [33] reported that a CLA isomer mixture suppressed MMP-2 and MMP-9 synthesis and activation in rat. Yang et al. [31] reported that *c*9,*t*11 CLA isomer reduced the type IV collagenase activity of SGC-7901 gastric carcinoma cells. The present study showed that the PMA-induced MMP-9 activity in colon cancer cells was significantly inhibited by *c*9,*t*11 CLA ($P<.05$) (Fig. 3), but *t*10,*c*12 CLA had no effect on MMP-9 activity. These results indicate that *c*9,*t*11 CLA is the effective isomer which can regulate MMP activities. Meyer et al. [27] reported that MMP-9 activity is regulated at the mRNA level in SW480 cells, but in this study, the reduction of PMA-induced MMP-9 activity was not accompanied by decreases in protein or mRNA levels (Figs. 4 and 5). Thus, more studies are needed to elucidate the mechanism by which *c*9,*t*11 CLA inhibits MMP-9 activity.

Several investigators have suggested that the chemopreventive effects of CLA may be due to eicosanoid production [34,35]. Liu and Belury [36] reported that the levels of arachidonic acid, a precursor for PGE_2, were consistently suppressed by dietary CLA, and Park et al. [37] showed that dietary CLA decreased eicosanoid levels in rat colonic mucosa. Varga et al. [38] reported that PGE_2 stimulates MMP synthesis and activation in fibroblasts. Therefore, a possible mechanism of the inhibition of MMP-9 activity by *c*9,*t*11 CLA in this study may be the reduction of PGE_2 synthesis.

Alternatively, CLA may inhibit MMP-9 activation by affecting the protein kinase C (PKC) pathway. In this study, we showed that PMA was capable of enhancing the cell migration and activity of MMP-9 in SW480 cells. In addition, Meyer et al. [27] showed that MMP-9 was activated by PMA in SW480 cells. Furthermore, Han et al. [28] reported that PMA caused an increase in MMP activity and in the in vitro invasion and motility properties of HM3 human colon cells. PMA has been reported to modulate diverse cellular responses through signal transduction pathways including the PKC pathway [39]. PKC is a member of the family of serine/threonine kinases that regulates a variety of cell functions including normal tissue function and repair and cancer cell migration [40]. Many studies suggest that PKC signaling pathways may be involved in modulating the invasive and metastatic properties of colon cancer cells [41,42]. It remains to be determined whether reduction of the PMA-induced migration and MMP activity by *c*9,*t*11 CLA is related to changes in the PKC pathway.

We also investigated the effect of CLA isomers on metastasis in vivo. Before performing the experiment with CT-26 cells, we had carried out several experiments to induce lung metastasis of SW480 cells in mice. However, SW480 cells did not yield tumors in the lungs of mice. The CT-26 cells utilized in our experiments have been widely used in experimental metastasis by other investigators [43,44]. The numbers of pulmonary nodules in mice fed the CLA isomer diets were significantly lower than those of mice fed a control diet ($P<.05$) (Fig. 6). There was no difference in the numbers of pulmonary nodules between the *c*9,*t*11 and *t*10,*c*12 groups. Hubbard et al. [45] reported that 0.5% and 1% CLA mixture significantly decreased mammary tumor metastasis in female BALB/cAnN mice. They also investigated the effect of CLA isomers on metastasis in mice and found that *c*9,*t*11 and *t*10,*c*12 CLA were equally effective in inhibiting mammary tumor

metastases in mice [46], which is consistent with the results obtained in the present study.

In the present study, the two CLA isomers were equally effective in reducing the number of pulmonary nodules in mice injected with colon cancer cells. However, in vitro studies showed that *c*9,*t*11 CLA but not *t*10,*c*12 CLA was effective in reducing cancer cell migration and MMP-9 activity. The present results cannot provide the explanation for the differences in the results between in vivo and in vitro studies. Perhaps, *t*10,*c*12 CLA may have decreased the number of pulmonary nodules by mechanisms not involved with changes in cell migration and MMP activity. In addition, previous results by other investigators have shown that *t*10,*c*12 CLA is more efficacious and potent in inhibiting colon cancer cell growth [17,18]. The ability of *t*10,*c*12 CLA to inhibit colon cancer cell growth may be the one of the reasons why *t*10,*c*12 CLA decreased the number of pulmonary nodules in the present study. More studies are needed to elucidate the mechanisms by which the individual CLA isomers inhibit pulmonary metastasis in mice.

In conclusion, we found that *c*9,*t*11 CLA is effective in inhibiting cell migration in a cell culture system and that both CLA isomers *c*9,*t*11 and *t*10,*c*12 reduce the numbers of pulmonary nodules in a mouse model. These results support the view that dietary strategies may prevent the metastasis and enhance the treatment of colon cancer patients, thus providing new therapeutic avenues.

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