Original Article

Antiproliferative effects of conjugated linoleic acid on human colon adenocarcinoma cell line Caco-2

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Conjugated linoleic acid (CLA) reduces body fat reserves, and reduces atherogenesis and type II diabetes in animal experiments. It has been reported that CLA have isomeric-specificity, such as *c9, t11* CLA with anticancer activity. The antiproliferative effects of two isomers of CLA (*c9, t11*-CLA, *t9, t11*-CLA) and their mixture on the human colon adenocarcinoma cell line Caco-2 were investegated in this paper. Caco-2 were incubated in serum-free medium. The antiproliferative effects of different concentrations (0, 25, 50, 100, 200μmol/L) of linoleic acid (LA) , *c9, t11*-CLA, *t9, t11*-CLA (the purity of LA and CLA was 96%) and a mixture of *c9, t11*-CLA and *t9, t11*-CLA (1:1 v/v) on caco-2 in various action time (1d, 2d, 3d, 4d) were tested in the present study. The antiproliferative effects of four substances in the same concentration and with the same action time were compared. All substances tested could inhibit Caco-2 cell proliferation. The higher anti-proliferation activity in the four materials is the mixture of CLA, then is t9,t11-CLA, c9,t11-CLA, and linoleic acid respectively. The activity is closely related to treatment time and concentration. The isomer *t9, t11*-CLA itself was found to have antiproliferative activity.

Key Words: conjugated linoleic acid, Caco-2, antiproliferation

Introduction

Conjugated linoleic acid (CLA) is the common denominator of a group of c18 fatty acids with two double bonds consisting of a mixture of positional and geometric isomers. CLA is a naturally occuring substance in food sources such as milk fat and the meat of ruminant animals, approximately 2.5-17.7 mg total CLA per gram fat. *c9, t11*-CLA is the main isomer in nature. CLA were first discovered by Pariza and his group when investigating the carcinogenic components of grilled beef.[1] Surprisingly, these modified fatty acids derived from the parent linoleic acid (18:2 n-6) were found to have anti-cancer rather than pro-cancer properties. Since these early discoveries an bewildering array of purported beneficial effects on health of CLA have been reported, mainly in animal models of human diseases and in cultures of various types of animal and human cells.

In the last decade, the biological activities of CLA were demonstrated by *in vivo* and *in vitro* tests, such as anticarcinogenesis, antiatherosclerosis, anabolism, and reduction of body weight and fat pad.[2-6] They also have reported beneficial regulatory effects on immune function, lipid and eicosanoid metabolism, cytokine and immuno-globulin production and can modulate the expression of a number of genes, either directly or through specific transcription factors involved in the many metabolic processes they affect. [7-12]

Environmental factors contribute to the development of many forms of cancer, and it has been estimated that 30% of all cases are affected by the diet, with dietary fat almost always floating to the top in this regard. In general, dietary fats may promote diverse forms of cancer indirectly through their caloric nutritional value, whereas polyunsaturatedfatty acids (PUFA) have additional direct effects oncarcinogenesis. Linoleic acid (LA, 18:2 n–6) is the most commonly PUFA.[13] Conjugated linoleic acid refers to a series of LA isomers that has an anticarcinogenic effect on various cells such as mammary gland, human hepatoma, forestomach and intestine.[14-17]But the mechanism by which CLA exerts the anticarcinogenesis effect has not been completely elucidated. Studies *in vitro* have shown that CLA inhibits the growth of human colon cancer cells, SW480 and HT-29 cells,[18-19] CLA also inhibited the growth of a human hepatoma cell line (HepG2), [20] human breast cancer cells MCF-7 *et al*. [14] The CLA preparations were used in these studies containing mixtures of many CLA isomers. Therefore, the effects of CLA monomer on colon cancer cell proliferation and the possible mechanisms by which they influence cancer cell proliferation remain unclear.

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The effects of different concentrations of linoleic acid, *c9, t11*-CLA, *t9,t11*-CLA and these two CLA mixture on the Caco-2 in various time was investigated in this study. The antiproliferative effects of four substances in the same concentration and the same action time were compared.

Methods

Matierals

Human colon adenocarcinoma cell line Caco-2 were from The Cell Bank, Shanghai Institute of Cell Biology. Heat-inactivated Fetal bovine serum, Dullbecco's modified Eagle's medium (DMEM), Trypsin-EDTA, penicillin and streptomycin were from Gibco BRL (Santa clara, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2, 5-dipheyltetrazolium bromide (MTT) was from Sigma Chemical Co. (St. Louis, Mo, USA). Serum-free Medium (SFM) was from Institute of Haematology, Jiangxi Medical College.

LA, *c9, t11*-CLA, *t9, t11*-CLA (the purity of LA and CLA were 96%) and the mixture of *c9, t11*-CLA and *t9, t11*-CLA (1:1, v/v) were prepared in our laboratory. All of these substances tested were dissolved in 96% ethanol. According to the cytotoxic test, the concentrations of 4 materials used in this research were 0, 25, 50, 100, 200μmol/L, and 0.1% ethanol was used as control. All solutions were made with bi-distilled, deionized and CO_2-free water.

Cell culture

Caco-2 cells between the 10 and 40 passages were used for the experiment. Cell were routinely maintained and subculture in 50 cm^2 plastic flasks at 37℃ in a humidified CO_2 incubator (95% air and 5% CO_2). The complete medium for cell maintenance was 4.5 g/L glucose DMEM containing L-glutamine, 10% FBS, 100u/mL penicillin, and 100 μg/mL streptomycin. When cells were 80% confluent, they were subcultured using 0.25% trypsin and 0.02% EDTA in D-Hanks' balanced salt solution (HBS), and the medium was changed every 48 h. All cultures contained ascorbic acid (50 ng/mL) and α-tocophenol phosphate (20 ng/mL) to protect fatty acids from oxidation. Prior to each experiment, cell number was determined with a cell counter based on conductivity measurement, and the viability of cells was assayed by the trypan blue dye exclusion test.

Substances tested

LA, *c9, t11*-CLA, *t9, t11*-CLA and the mixture of *c9, t11*-CLA and *t9, t11*-CLA (1:1, v/v) were used in this study. For each substance, four concentrations of 0, 25, 50, 100, 200μmol/L were used to test the concentration effect, and four period of 1, 2, 3, 4 days to test the time effect.

MTT assay

MTT is a tetrazolium salt that can be cleaved by mitochondrial dehydrogenase in living cells but not dead one to give a dark blue product measured with spectrometer. The effects of LA, *c9, t11*-CLA, *t9, t11*-CLA and the mixture of *c9, t11*-CLA and *t9, t11*-CLA on cell growth were tested by MTT method. On day 0, cells were seeded in 96-well plates at a density of 1.0×10^4 cells per well. To determine the effects of the four materials better, On day 2, the original growth medium was removed and replaced with serum-free medium containing various concentrations of the substances tested (0-200 μmol/L) dissolved in ethanol. Although 0.1% ethanol in the medium had no detectable effects on cell growth, the same concentration of ethanol was also added to the controls. Caco-2 cell was cultured for 1, 2, 3, 4 days continuously, then, the medium was discarded, 20 μl MTT solution (0.5 mg/mL in PBS) was added to all wells, and the cells were incubated for 4h at 37℃ in a humidified CO_2 incubator (95% air and 5% CO_2). 100 μl DMSO was added to each well to dissolve water insoluble MTT-formazan crystals. The plates were transferred to microplatereader. Absorbance was recorded at 570 nm. All experiments were performed at least 4 times with 4 wells of each concentration.

Statistics

Values were expressed as mean ± S.D. One-way ANOVA was performed with the Statistics Analysis System (SAS Users Guide Statistics, SAS Institute, Cary, NC). The statistical significance of the differences was assumed at $p<0.05$ (*).

Results and discussion

The increasing incidence of colorectal cancer, already one of the most prevalent cnacers in the West, makes urgent the development of strategies to prevent this disease. With regard to prevention, diet and non-steroidal anti-inflammatory drugs have drawn considerable attention in recent years.[21] Cancer is frequently described as a disorder of the balance between cell proliferation and cell death. A better understanding of the intrinsic differences between normal and cancer cells should lead to the development of more effective therapeutic interventions for the prevention of tumor progression. Earlier *in vitro* studies indicated that CLA inhibited colon cancer cell growth (Caco-2, HT-29, SW480, *et al*).[22-25] Studies in vitro tend to support the anti-cancer effects of CLA and show that they inhibit cell proliferation, inhibit eicosanoid formation and induce apoptosis by modulating p53 and bcl-2 expression in human prostate cancer (PC3) cell lines.[26] The

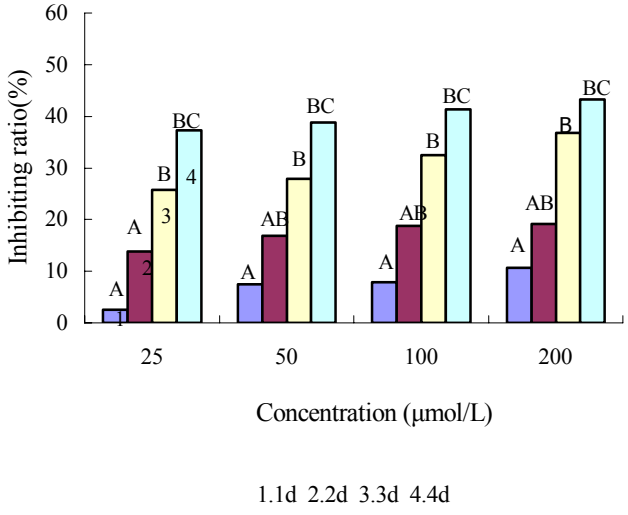

Figure1. Effect of LA of the same concentration on Caco-2 cell growth in different time. *The significant different of concentrations ($p<0.05$). Values with different letters were significant different in time ($p<0.05$). Values with the same letter were no significant different.

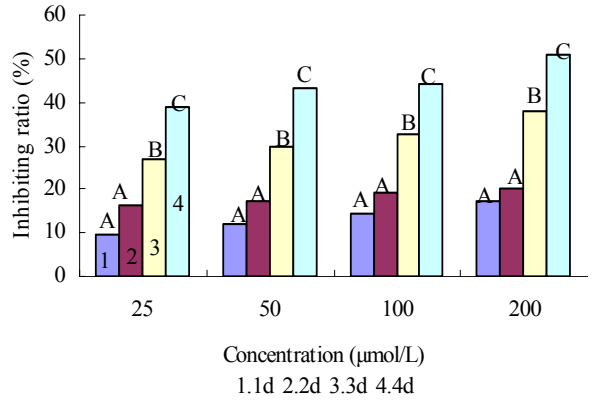

Figure 2. Effect of c9,t11-CLA of the same concentration on Caco-2 cell growth in different time. *The significant different of concentrations (p<0.05). Values with different letters were significant different in time (p<0.05). Values with the same letter were no significant different.

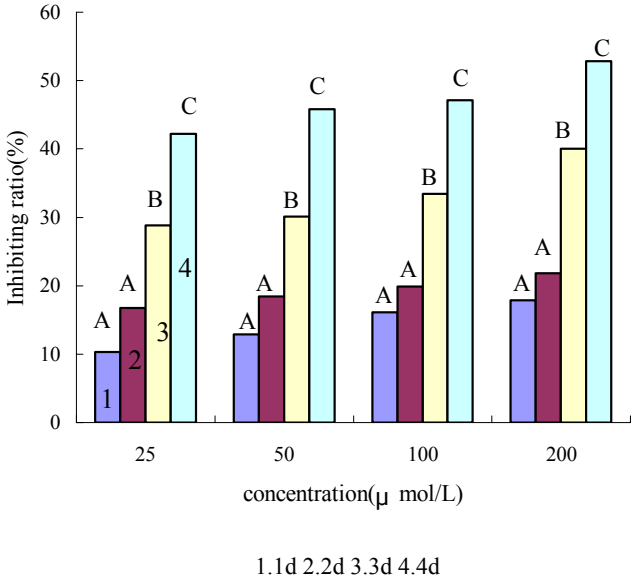

Figure 3. Effect of t9,t11-CLA of the same concentration on Caco-2 cell growth in different time. *The significant different of concentrations (p<0.05). Values with different letters were significant different in time (p<0.05). Values with the same letter were no significant different.

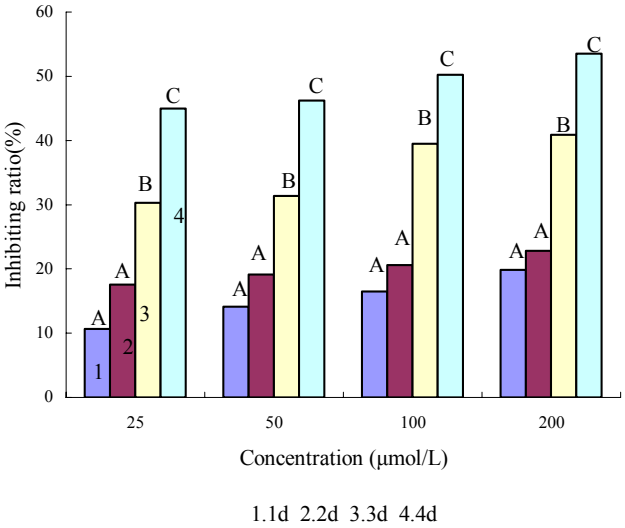

Figure 4. Effect of the mixture of CLA of the same concentration on Caco-2 cell growth in different time. *The significant different of concentrations (p<0.05). Values with different letters were significant different in time (p<0.05). Values with the same letter were no significant different.

others report that the two main isomers of CLA elicit different effects on eicosanoid production and oncogene regulation to induce apoptosis. The *t10, c12*-CLA isomer affected mainly eicosanoid formation and the COX-2 enzyme whilst *c9,t11*-CLA affected the expression of oncogenes. Similarly, Palombo *et al*. have shown that both isomers of CLA have anti-proliferative and pro-apoptotic effects on hum an prostate cancer cells. [27] CLA isomers elicited signiffcant anti-proliferative effects on human HT-29 colorectal cancer cells *in vitro* through inhibition of DNA synthesis and induction of apoptosis. [24] CLA inhibited the heregulin-a-induced phosphorylation of ErbB2 and ErbB3, recruitment of p85 subunit of PI3-kinase to the ErbB3 receptor, ErbB3 associated PI3-kinase activities and phosphorylation of Akt. CLA decreased ErbB2 and ErbB3 mRNA and protein expression in a dose-dependent manner.[24, 28]

To assay antiproliferative effects of four materials, MTT assay was used to provides a quantitative determination of

Table 1. Effect of all substances in the same concentration on Caco-2 cell at 1d

Substances tested	Concentration(μmol/L)				
	control	25	50	100	200
LA	1.33±0.1^{z}	1.30±0.23^{z}	1.23±0.17^{z}	1.23±0.23^{z}	1.19±0.22^{a,b}
c9,t11-CLA	1.33±0.1	1.20±0.15^{a}	1.17±0.12^{a,b}	1.14±0.10^{a,b}	1.11±0.09^{a,b}
t9,t11-CLA	1.33±0.1	1.20±0.13^{a,b}	1.16±0.09^{a,b}	1.12±0.10^{a,b}	1.10±0.04^{a,b}
the mixture CLA	1.33±0.1	1.19±0.13^{a,b}	1.15±0.14^{a,b}	1.11±0.09^{a,b}	1.07±0.07^{a,b,}

*The significant different of concentrations (p<0.05). Values with different letters were significant different in time (p<0.05). Values with the same letter were no significant different.

Table 2. Effect of all substances in the same concentration on Caco-2 cell at 2d

Substances tested	Concentration (μmol/L)				
	control	25	50	100	200
LA	1.33±0.08^{a}	1.15±0.05^{b}	1.11±0.05^{b}	1.08±0.06^{b}	1.07±0.13^{b}
c9,t11-CLA	1.33±0.08	1.12±0.08^{b}	1.10±0.17^{b}	1.07±0.08^{b}	1.06±0.10^{b}
t9,t11-CLA	1.33±0.08	1.11±0.18^{b}	1.08±0.15^{b}	1.07±0.06^{b}	1.04±0.18^{b}
the mixture CLA	1.33±0.08	1.10±0.12^{b}	1.08±0.11^{b}	1.06±0.14^{b}	1.03±0.07^{b}

*The significant different of concentrations (p<0.05). Values with different letters were significant different in time (p<0.05). Values with the same letter were no significant different.

Table 3. Effect of all substances in the same concentration on Caco-2 cell at 3d

Substances tested	Concentration (μmol/L)				
	control	25	50	100	200
LA	1.33±0.28[a]	0.98±0.08[b]	0.96±0.13[b]	0.90±0.15[b]	0.84±0.13[b]
c9,t11-CLA	1.33±0.28	0.97±0.12[b]	0.93±0.23[b]	0.89±0.18[b]	0.82±0.13[b]
t9,t11-CLA	1.33±0.28	0.94±0.10[b]	0.93±0.17[b]	0.88±0.22[b]	0.80±0.21[b]
the mixture CLA	1.33±0.28	0.92±0.07[b]	0.91±0.14[b]	0.80±0.17[b]	0.78±0.19[b]

*The significant different of concentrations (p<0.05). Values with different letters were significant different in time (p<0.05). Values with the same letter were no significant different.

Table 4. Effect of all substances in the same concentration on Caco-2 cell at 4d

Substances tested	Concentration (μmol/L)				
	control	25	50	100	200
LA	1.32±0.38[a]	0.83±0.16[b]	0.81±0.26[b]	0.77±0.22[b]	0.75±0.23[b]
c9,t11-CLA	1.32±0.38	0.81±0.27[b]	0.75±0.24[b]	0.74±0.16[b]	0.65±0.16[b]
t9,t11-CLA	1.32±0.38	0.76±0.33[b]	0.72±0.30[b]	0.70±0.23[b]	0.62±0.21[b]
the mixture CLA	1.32±0.38	0.73±0.33[b]	0.71±0.38[b]	0.66±0.32	0.61±0.21[b]

*The significant different of concentrations (p<0.05). Values with different letters were significant different in time (p<0.05). Values with the same letter were no significant different.

metabolically active cells in present study. Caco-2 cells were exposed to various concentrations of four materials for different times. The effects of LA, c9,t11-CLA, t9,t11-CLA, mixture of CLA on caco-2 cell growth were tested in different concentrations and times, the results were shown in the figure1-4 and in the table 1-4. The different concentrations (0, 25, 50, 100, 200 μmol/L) of all substances tested could inhibite the Caco-2 cell proliferation in various time (1d，2d，3d，4d) in comparison to the controls. The antiproliferative effects of four materials were closely related to the treatment time and the concentration. when substances tested was present continuously in the medium at the same concentration, Caco-2 cell growth was inhibited in a time-dependent manner, the inhibiting ratio were significantly different in various time (p<0.05). When substances tested was present continuously in the medium in the same time, Caco-2 cell growth was inhibited in a concentration-dependent manner, however the inhibiting ratio were not significantly different at different concentration.

The present study has shown that all substances tested could inhibit Caco-2 cell growth, and the higher antiproliferative activity in the four materials is the mixture of the CLA (t9, t11-CLA, c9, t11-CLA), then is t9, t11-CLA, c9,t11-CLA, and LA, respectively. According to our knowledge, the anticarcinogenic activity of t9, t11-CLA have not been reported now. The inhibitory ratio of the four materials at the same time were not significantly different at different concentrations. The four materials tested inhibited Caco-2 cells growth in dose-dependent and time-dependent manner. And the strongest of the antiproliferative activities in the four materials was the mixture of CLA and t9, t11-CLA.

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