Effect of cis-9,trans-11-conjugated linoleic acid on cell cycle of gastric adenocarcinoma cell line (SGC-7901)

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INTRODUCTION

Gastric cancer is common in China[11-12], and it is currently thought to be caused by environmental factors, with diet being an important modifying agent[16-17]. Its mechanism of prevention and treatment still makes it become a hot spot in this area[18-19]. Its anticancerous potential Dietary fat has been implicated as an enhancing agent in carcinogenesis by both epidemiological and animal studies. Consumption of meat, specifically animal fat, has been implicated in a number of disease processes[20-22]. However, several epidemiological studies have suggested an association between increased consumption of meat and fat and decreased risk of stomach, mammary and esophageal cancers[21,24]. Among the fatty acids, only the essential fatty acid, linoleic acid (LA), has been clearly shown to enhance mammary tumorigenesis[21]. However, isomeric derivatives of cis-9,cis-12-octadecadienoic acid (linoleic acid, LA) containing a conjugated double-bond system (conjugated linoleic acid, CLA) showed inhibitory effect on carcinogenesis in animal studies[23,24]. CLA has a mixture of positional (9/11 or 10/12 double bonds) and geometric (various cis/trans combinations) isomers of LA formed by rumen and colon bacteria. The ability of CLA to prevent mammary and other tumors in rodents has been identified and has been the subject of several reviews[25]. There are eight potential isomers of CLA, but the cis-9, trans-11 and trans-9, cis-11 isomers are thought to be active as potential antioxidant and anticarcinogenic agents. Therefore, it is of interest to investigate more extensively the anticancer activities of CLA.

In the present study, we investigated the effect of cis-9, trans-11-CLA(cis-9,11-CLA) on the cell cycle of human gastric adenocarcinoma cells (SGC-7901).

MATERIALS AND METHODS

Materials
c9,11-CLA, a monoisomer of c-9,11-octadecadienoic acid with 98% purity, was obtained from Dr. Rui-Hai Liu(Food Science and Toxicology, Department of Food Science, Cornell University, Ithaca, NY, USA). The c9,11-CLA was dissolved in 96ml·L⁻¹ ethanol, and was diluted to the following concentrations: 0.25, 50, 100 and 200µmol·L⁻¹.

Methods

Cell culture Human gastric adenocarcinoma cells(SGC-7901), purchased from Cancer Research Institute of Beijing (China), were cultured in RPMI 1640 (Gibco) medium, supplemented with calf serum 100ml·L⁻¹, penicillin (100×10³u·L⁻¹) and streptomycin (100mg·L⁻¹). The pH was maintained at 7.2-7.4, by equilibration with 5% CO₂. The temperature was maintained at 37°C. The cells were sub-cultured with a mixture Ethylenedinitrile tetraacetic acid (EDTA) and trypsin.

Cell growth curve The SGC-7901cells were seeded in six 24 well plates (Nuc,Co.); each well contained 2×10⁶ cells. After 24h, the medium of different plates was replaced with media supplemented with c9, 11-CLA at different concentrations. On the next day, the numbers of cells of 3 wells from each plate were determined daily by using the trypan blue staining. The means were obtained on each of
eight days and were used to draw a cellular growth curve. The inhibitory rate(IR) on the 8th day was calculated, as follows:

\[ \text{IR(\%)} = \frac{\text{Total number of cells in negative control (8d)} - \text{Number of cells in test groups(8d)}}{\text{Total number of cells in negative control(8d)}} \times 100\% \]

[\[^3\text{H}\text{-labeled precursor incorporation}\] SGC-7901 cells (5x10^4/well in 24 well plate) were cultured in appropriate medium for 24h prior to beginning the experiment. The medium was, then, replaced with different concentrations of c9, t11-CLA. After 18 and 42h, the cells were incubated with [\[^3\text{H}\] thymidine (China Nucleus Institute, 0.5μCi/mL, 1.0μCi/well). After 6h the cells were harvested with trysin/EDTA. Cells were collected in an acetic fiber filter with cellular collector and washed three times with PBS. The filter was dried overnight at 37°C. The filter was transferred into liquid of scintillation (containing 1% po normal goat serum in PBS to block non-specific binding. The sections were deparaffinized in xylene and rehydrated through graded alcohol. The sections were incubated for 10min at 95°C in 10mmol·L⁻¹ sodium citrate (pH 6.0) buffer for PCNA staining. Endogenous peroxidases were inactivated by immersing the sections in hydrogen peroxidase solution. The sections were incubated with biotinylated anti-mouse or anti-rabbit IgG (Zhongshan Co. China) for 30min. The chromogenic reaction was developed with DAB (diaminobenzidine) for 10min, and all sections were counterstained with hematoxylin. Controls consisted of omission of the primary antibody. The Positive Rate(PR) was calculated as follows:

\[ \text{PR(\%)} = \frac{\text{Number of positive cells}}{\text{Total number(2x10^5)}} \times 100\% \]

### RESULTS

#### Effect of c9,t11-CLA on SGC-7901 cell growth

As shown in Figure 1, Growth of the cells in various concentrations of c9, t11-CLA did not differ from the negative control within 3d. After 3d, SGC-7901 cells incubated in 25 and 50μmol·L⁻¹ of c9,t11-CLA grew at a lower rate than the negative control. While in 100 and 200μmol·L⁻¹ concentrations of c9, t11-CLA, cell proliferation was significantly inhibited. The inhibitory rate of various c9, t11-CLA concentrations were 5.9%, 20.2%, 75.6% and 82.4%, respectively.

![Figure 1](image1.png)  
**Figure 1** Growth curve of SGC-7901 cells cultured in various concentration of c9, t11-CLA

#### Effect on DNA synthesis

The effect of CLA on isotope incorporation into SGC-7901 cells are presented in Table 1. SGC-7901 cells preincubated in media supplemented with various c9, t11-CLA concentration (except for 25μmol/L, 24h) incorporated significantly less \[^3\text{H}\]Tdr than did the negative control (P<0.05 and P<0.01, Table 1). The inhibitory rate (IR) displayed a dose-response relationship as the concentration of c9, t11-CLA increased.

![Figure 2](image2.png)  
**Figure 2** Expression of PCNA on SGC-7901 cells treated with c9, t11-CLA

#### Cell proliferation

As shown in Figure 2, expression raues of PCNA (Figure 3.1) on SGC-7901 cells gradually decreased after SGC-7901 cells were incubated with different concentrations of c9,t11-CLA at various times. Moreover, SGC-7901s cell expressed significantly less PCNA than did the negative control (P<0.01). The expression rate displayed a dose-response relationship as the concentrations of CLA increased.

### Table 1

| \((\text{c9,t11-CLA (μmol/ L)}\times 10^3)\) | \(^{3}\text{H}\text{-Tdr incorporation (cpm, tso)}\) | Inhibitory Rate (%) |
|------------------------------------------|------------------------------------------|-------------------|
| 0                                       | 2165±172                                 | -                 |
| 10                                      | 2065±261                                 | 78.2              |
| 50                                      | 2206±291                                 | 42.7              |
| 100                                     | 2096±3498                                | 57.8              |
| 200                                     | 472±2600                                 | 88±15             |

\[^{3}\text{P}<0.05, ^{3}\text{P}<0.01\ vs \text{negative control}\]
Expressions of cyclin A, B1, and D1 and p16\textsuperscript{ink4a}, p21\textsuperscript{waf1}

The expression of cyclin A, B1, and D1 (Figure 3.2-4) on SGC-7901 cells was decreased (Table 2) after SGC-7901 cell were incubated with different concentrations of 9,11-CLA for 24h and 48h while cyclin-dependent kinases inhibitors (P16\textsuperscript{ink4a}, and P21\textsuperscript{waf1}) increased (Table 3, Figure 3.5-6).

| 9,11-CLA (µmol/L) | 24h | 48h |
|-------------------|-----|-----|
|                   | Cyclin A | Cyclin B | Cyclin D1 | Cyclin A | Cyclin B | Cyclin D1 |
| 0                 | 10.7 | 4.2 | 9.5 | 5.9 | 5.1 | 6.0 |
| 25                | 11.0 | 4.8 | 3.6\textsuperscript{b} | 8.5 | 5.5 | 3.7\textsuperscript{b} |
| 50                | 7.9 | 2.5 | 3.5\textsuperscript{b} | 5.0 | 3.1\textsuperscript{b} | 3.7\textsuperscript{b} |
| 100               | 4.4\textsuperscript{b} | 2.6\textsuperscript{b} | 2.1\textsuperscript{b} | 1.3\textsuperscript{b} | 0.7\textsuperscript{b} | 0.6\textsuperscript{b} |
| 200               | 2.3\textsuperscript{b} | 1.8\textsuperscript{b} | 0.4\textsuperscript{b} | 0.5\textsuperscript{b} | 0.6\textsuperscript{b} | 0 |

\textsuperscript{b}P<0.01 vs negative control

Table 3 Positive rates of p16\textsuperscript{ink4a} and p21\textsuperscript{waf1} on SGC-7901 cells treated with 9,11-CLA(%)

| 9,11-CLA (µmol/L) | 24h | 48h |
|-------------------|-----|-----|
|                   | p16\textsuperscript{ink4a} | p21\textsuperscript{waf1} | p16\textsuperscript{ink4a} | p21\textsuperscript{waf1} |
| 0                 | 1.0 | 0.2 | 0.8 | 0.6 |
| 25                | 0.7 | 1.4\textsuperscript{b} | 0.2 | 0.8 |
| 50                | 1.4 | 1.0\textsuperscript{b} | 3.0\textsuperscript{b} | 2.5\textsuperscript{b} |
| 100               | 2.8\textsuperscript{b} | 4.1\textsuperscript{b} | 4.6\textsuperscript{b} | 3.8\textsuperscript{b} |
| 200               | 3.6\textsuperscript{b} | 5.2\textsuperscript{b} | 5.0\textsuperscript{b} | 6.3\textsuperscript{b} |

\textsuperscript{b}P<0.01 vs negative control
Discussion

CLA is a naturally occurring fatty acid in animal’s food. Dietary sources of CLA include grilled beef, cheese, and related foods. Another source of CLA is its endogenous generation via the carbon center free radical oxidation of linoleic acid[40]. Over the past ten years, a number of research works of animal experiments have supported the observation that CLA is an effective chemopreventive agent of cancer, and that it can inhibit carcinogenesis of different tissues at different stages of induction by chemical agents[41,42]. Several investigators in our group have reported that c9,t11-CLA is an effective agent to prevent carcinogenesis[43,44] and cancer[45-47]. Zhu’s study[48] demonstrated that c9,t11-CLA could significantly inhibit the mouse forestomach neoplasia induced by B(a)P(50mg·kg-1) in post-initiation in short term(23weeks). The incidences of tumors of mice in the B(a)P group, B(a)P with high dose CLA(5μL·g-1) group and B(a)P with low dose CLA(2.5μL·g-1) group were 100%, 60% and 69% respectively (P<0.05). Xue’s research[49] also indicated that the incidence of neoplasm in mouse forestomach in the B(a)P group,75% pure c9,t11-CLA group, 98% pure c9,t11-CLA group and 98% pure t10,c12-CLA group were 100.0%, 75.0%, 69.2%, and 53.8%, respectively. This maybe due to an inhibition mitogen of activated protein kinase(MAPK)-a way to reduce carcinogenesis. The data from our research group suggested that c9,t11-CLA could inhibit proliferation of cancer cells, i.e. SGC-7901 cells[50] and MCF-7 cells[51,52], and induced cancer cell (SGC-7901) apoptosis[53]. Moreover, the inhibiting effect of c9,t11-CLA on SGC-7901 cell proliferation may be related to cell cycle.

As shown in Figure 1, c9,t11-CLA at various concentrations in 8 days reduced the proliferative activity of SGC-7901 cells and its inhibitory rates were from 5.92% to 82.44%, but the mechanism of inhibition of cell proliferation of SGC-7901 cells treated with various concentrations c9,t11-CLA for 24h and from 14.0% to 97.5% after 48h displayed a dose-response relationship. In the meantime, we investigated further the expressions of PCNA and protein cyclin-dependent kinase inhibitors (CDKI) on SGC-7901 cells treated with various concentrations of c9,t11-CLA. PCNA (proliferating cell nuclear antigen) plays an essential role in both the replication and repair of DNA, and is an essential component of the DNA replication machinery, acting as the processing factor for polymerases and. In addition to its role in replication, PCNA is not only required for base excision-repair of nucleotides, but also binds to cell cycle regulatory proteins such as p21 and Gadd45. In this study, we discovered that the expression of PCNA on SGC-7901 cells gradually decreased with increasing concentrations of c9,t11-CLA in comparison with negative controls(shown in Figure 2). In other words, DNA replication lessens, thereby resulting in slower on SGC-7901 cell proliferation.

The fundamental task of the cell cycle is to ensure that DNA is faithfully replicated once during S phase and that identical chromosomal copies are distributed equally to two daughter cells during M phase. The cell cycle is a complex process, regulated by many factors, which can be divided into three groups: cyclins(A,B, D,E,....,H); cyclin-dependent kinases (CDK, including CDK4-, CDK2-); CDK inhibitors (CDKI, including p16 family and p21 family). They are balanced through mutual interactions. Uncontrolled cell proliferation is the hallmark of cancers which are the result of damage to genes that directly regulate their cell cycles. Using immunocytochemical technique to detect expressions of cyclins and CDKI, we demonstrated that the expressions of cyclin A, B, and D1 on SGC-7901 cells treated with various concentrations c9,t11-CLA were reduced, whereas expressions of CDKI(p16ink4a and p21war1) increased, as compared with those of negative controls. Successive actions of CDKs promote cell-cycle progression in mammalian cells. Various cyclins bind and activate CDKs at specific times during the cell cycle.

Mammalian cyclin A activates CDK2[54] in S-phase and CDK1 (Cdc2) in G2- and M-phases. One important mechanism that enables sequential activation of cyclin-CDK complexes is the periodic synthesis and destruction of cyclins. Cyclin A expression starts late in G2-phase and is increasing through S- and G1-phase before the protein is degraded in M-phase. The cell cycle-dependent expression of cyclin B is critical for the proper timing of a cell’s entry into mitosis which is dependent both upon the binding of CDK2 to cyclin B, as well as a series of phosphorylation and dephosphorylation events. The cyclin B1 protein accumulates during interphase and peaks at the G2-M phase transition[55]. One of the crucial substrates of G2 phase cyclin, including CDK4, in the complex with D-type cyclins(cyclin D1, D2 and D3), is Rb protein (pRb), which is the product of the retinoblastoma susceptibility gene. Rb protein plays an important role in the regulation of the G1 to S phase transition in normal cells and the function of pRb is regulated by phosphorylation. Thus, during the G1 and G2 phase, Rb protein is in an- or underphosphorylated state and binds to E2F family transcription factors. Cyclin Ds/CDK4 becomes activated around the mid G1 phase, resulting in the accumulation of increasingly phosphorylated, inactive forms pRb. This causes the release of E2F family transcription factors which induce the expression of S-phase genes by positive regulation through E2F-binding sites[see Figure 4] [56]. It is also known that abrogation of the functions of cyclin A prevents entry into the S phase. From the beginning of the S phase Rb protein remains in the hyperphosphorylated inactive state until the end of M phase; such condition is thought to be due to both cyclin A/CDK2 and cyclin A/B/Cdc2 in catalyzing the phosphorylation reaction[57]. p16ink4a is the founder member of a family of proteins with the ability to inhibit CDK4 and the CDK4-related kinase CDK6.

The INK4 family is composed of four members in mammalian organisms: p16ink4a, p15ink4b, p18ink4c, and p19ink4d. The four mammalian INK4 proteins have similar biochemical properties: all of them bind to CDK4 and CDK6 and inhibit the kinase activity of the CDK4/cyclin D complexes(Figure 4)[58]. The INK4 inhibitor causes G1 arrest indicating that the phosphorylation of pRb on residues specific for CDK4( and possibly CDK6) is critical for G1/S progression. While p21cip1/waf1 family, comprising p21(cip1/waf1), p27(cip1) and p57(cip2), bind to a variety of CDKs and cyclins, preferentially to cyclin/CDK complexes rather than monomeric forms and also inhibit performed active cyclin/CDK complexes[see Figure 4][59]. In addition to its role as a CDK1, p21cip1/waf1 has been shown to block DNA replication by direct interaction with PCNA mentioned above. However, p21cip1/waf1 does not inhibit the PCNA-dependent nucleotide excision-repair of DNA. In deed, DNA damage leads to an increase in the level of p53, and result in p21-mediated cell cycle arrest in the G1 phase, which persists
until DNA repair is completed\(^{[44]}\). Thus, it is proposed that p21\(^{Cyclin}\text{p}1\) plays an important role under such conditions as terminal differentiation and cell senescence.

In conclusion, c9-r11-CLA may inhibit cell growth and proliferation by a decrease in the expressions of cyclin A, B, and D, and an increase in that of CDKp at 16\(^{\text{p}}\) cells and p21\(^{Cyclin}\text{p}1\) on SGC-7901 cells in comparison with the negative controls. This result suggested that the inhibition effect of c9-r11-CLA on SGC-7901 cell proliferation is related to the cell cycle. The whole mechanism of the action of c9-r11-CLA on SGC-7901 cell cycle further research.

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Edited by Lu HM