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Original Article

Effects of *cis*-9, *trans*-11-Conjugated Linoleic Acid on Cancer Cell Cycle

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ABSTRACT

Objectives: To determine the effect of cis-9, trans-11-conjugated linoleic acid on the cell cycle of mammary cancer cells (MCF-7) and its possible mechanism of inhibition cancer growth.

Methods: Using cell culture and immunocytochemical techniques, we examined the cell growth, DNA synthesis, expression of PCNA, cyclin A, B_1, D_1, $p16^{ink4a}$ and $p21^{cip/waf1}$ of MCF-7 cells which were treated with various c9, t11-CLA concentrations (25 mM, 50 mM, 100 mM and 200 mM) of c9, t11-CLA for 24 and 48 h, with negative controls (0.1% ethanol).

Results: The cell growth and DNA synthesis of MCF-7 cells were inhibited by c9, t11-CLA. MCF-7 cells, after treatment with various c9, t11-CLA doses mentioned above for 8 days, the inhibition frequency was 27.18%, 35.43%, 91.05%, and 92.86%, respectively and the inhibitory effect of c9, t11-CLA on DNA synthesis (except for 25 mM, 24 h) incorporated significantly less ^{3}H-TdR than did the negative control ($P<0.05$ and $P<0.01$). To further investigate the influence on the cell cycle progression, we found that c9, t11-CLA may arrest the cell cycle of MCF-7 cells. Immunocytochemical staining demonstrated that MCF-7 cells preincubated in media supplemented with different c9, t11-CLA concentrations at various times significantly decreased the expressions of PCNA, and Cyclin A, B_1, D_1 compared with the negative controls ($P<0.01$), whereas the expressions of $p16^{ink4a}$ and $p21^{cip/waf1}$, cyclin-dependent kinases inhibitors (CDKI), were increased.

Conclusions: The cell growth and proliferation of MCF-7 cells is inhibited by c9, t11-CLA by blocking the cell cycle, which reduces expressions of cyclin A, B_1, D_1 and enhances expressions of CDKI ($p16^{ink4a}$ and $p21^{cip/waf1}$).

Key words: mammary adenocarcinoma cells (MCF-7), *cis*-9, *trans*-11-conjugated linoleic acid (c9, t11-CLA), immunocytochemistry, cell cycle, inhibition

Introduction

The majority of human cancers are suggested thought to be caused by environmental factors (1), with diet being an important modifying agent (2). Dietary fat has been implicated as an enhancing agent in carcinogenesis by both epidemiological evidence and animal studies. Consumption of meat and specifically animal fat, has been implicated in a number of disease processes. However, several epidemiological studies have suggested an association between increased consumption of meat and fat and a decreased risk of stomach (3, 4) and esophageal cancers (5, 6). Among the fatty acids, only the essential fatty acid, linoleic acid (LA), has been clearly shown to enhance mammary tumorigenesis (7). However, isomeric derivatives of *cis*-9, *cis*-12-octadecadienoic acid (linoleic acid, LA) containing a conjugated double-bond system (conjugated linoleic acid, CLA) has shown inhibitory carcinogenesis in animal studies (8–10). 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 colonic bacteria. The ability of CLA to prevent mammary and other tumors in rodents has been identified and was the subject of several reviews (11, 12). There are eight potential isomers of CLA, but the *cis* 9, *trans* 11 and *trans* 9, *cis* 11 isomers were suggested 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 (c9, t11-CLA) on the cell cycle of human mammary

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adenocarcinoma cells (MCF-7).

Materials and Methods

Cell and CLA reagent

Human mammary adenocarcinoma cells (MCF-7), purchased from Cancer Research Institute of Beijing (China), were cultured in DMEM (Gibco Company) medium, supplemented with calf serum 10%, penicillin (100 units/ml) and streptomycin (100 μg/ml). The pH was maintained at 7.2～7.4, by equilibration with 5% CO_2. The temperature was maintained at 37°C. The cells were subcultured with a mixture of 0.02% Ethylenedinitrile tetraacetic acid (EDTA) and 0.2% trypsin.

c9, t11-CLA, a monoisomer of c-9, t11-octadecadienoic acid, was obtained from Dr. Ruihai Liu (Food Science and Toxicology, Department of Food Science, Cornell University Ithaca, NY, USA); the purity of this material was 98%. The c9, t11-CLA was dissolved in 96% ethanol. The samples were diluted several times with concentrations of ethanol in accordance with the frequency of cell survival. The concentrations were as follows: 0, 25 μM, 50 μM, 100 μM and 200 μM.

Cell growth curve

The MCF-7 cells were seeded in six 24 well plates (nuc, Co.); each well containing 2×10^4 cells. After 24 h, the medium of different plates was replaced with media supplemented with c9, t11-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 frequency (IF) on the 8th day was calculated, as follows:

$$\text{IF (\%)}=\frac{\text{Total number of cells in negative controls (8d)}-\text{Number of cells in test groups (8d)}}{\text{Total number of cells in negative controls (8d)}}\times100\%$$

[^{3}H]- labeled precursor incorporation

MCF-7 cells (5×10^4/well in 24-well plates) were cultured in appropriate medium for 24 h prior to beginning the experiment. The medium was, then, replaced with different concentrations of c9, t11-CLA. After 18 h and 42 h, the cells were incubated with [^{3}H] thymidine (China Nucleus Institute, 0.5 μci/ml, 1.0 μci/well). After 6 h the cells were harvested with a mixture of trypsin/EDTA as mentioned above. 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 for scintillation (containing 1% po and 2% pop in xylene) and cpm values were determined using a liquid Scintillation Counter (LS6500, Beckmen Co.).

Cell cycle analysis

MCF-7 cells (5×10^5 cells in 25 ml bottles) were seeded in appropriate medium for 24 h prior to beginning the experiment. The medium was then replaced with different concentrations c9, t11-CLA. After 24 h and 48 h, the cells were harvested using trypsin/EDTA, washed twice with cold PBS, fixed in 70% ethanol on ice for 30 minutes, and washed again. Cells were then stained by adding 1 ml of PI mixture (containing 50 μg propidium iodide, 0.2 mg RNAse, 5 μl Triton X-100, and 1 mg citromalic acid) in the dark (4°C, 30 minutes). Cell cycle analysis was subsequently performed by flow cytometry using a FACSCalibur Analyzer (BD Biosciences) with a 15-milliwatt air-cooled argon laser (excitation=488 nm). DNA content in phases of the cell cycle was analyzed using ModiFix LT software.

Cell samples

MCF-7 cells samples were pellet treated at 24 h and 48 h by various concentrations of c9, t11-CLA and collected by centrifugation. Specimens were fixed immediately in 4% formaldehydum polymerisatum and embedded in paraffin. Mammary cancer tissue from a patient who had been operated upon served as a reference.

Primary antibodies

To examine the proliferating cell nuclear antigen (PCNA) in cell proliferation and to determine cyclins (A, B_1 and D_1) and cyclin-dependent kinases inhibitors ($p16^{ink4a}$ and $p21^{waf1}$) in the cell cycle of MCF-7 cells, we used six primary antibodies: corresponding mouse monoclonal antibodies for cyclin B_1 and D_1, PCNA and $p21^{waf1}$ and corresponding rabbit polyclonal antibodies for cyclin A and $p16^{ink4a}$. PCNA, $p16^{ink4a}$, and cyclin D_1 were purchased from Calbiochem Co., USA; others were from Zhongshan Co., China.

Immunocytochemistry

Immunocytochemical staining was performed on serial sections at room temperature, using the horseradish peroxidase method. The sections were deparaffinized in xylene and rehydrated through graded alcohol. The sections were incubated for 10 minutes at 95°C in 10 mM sodium citrate (pH 6.0) buffer for PCNA staining. Endogenous peroxideses were inactivated by immersing the sections in hydrogen peroxide for 10 minutes, and then incubated for 10 minutes with 10% normal goat serum in PBS to block non-specific binding. The sections were subsequently incubated overnight at 4°C with relevant antibodies (1 : 50 dilution respectively). The next day, the sections were incubated with biotinylated anti-mouse or anti-rabbit IgG (Zhongshan Co., China) for 30 minutes, followed by peroxidase-conjugated streptavidin (Zhongshan Co., China) for 30 minutes. The chromogenic reaction was developed with DAB (diaminobenzidine) for 10 minutes, and all sections were counterstained with hematoxylin. Controls consisted of omission of the primary antibody. The Positive Frequency (PF) was calculated as follows:

$$\text{PF (\%)}=\frac{\text{The number of positive cells}}{\text{Total number }(2\times10^4)}\times100$$

Statistical analysis

Analysis of data was performed using Student's *t* test or X^2 test. A value of $P<0.05$ was considered to be statistically significant.

Results

Effect of c9, t11-CLA on MCF-7 cell growth

The inhibitory effect of c9, t11-CLA on the growth of MCF-7 cells is shown in Fig. 1. MCF-7 cells grew rapidly before the second day in the culture media supplemented with 25 μM and 50 μM c9, t11-CLA and in the negative control. After 3 days, the number of MCF-7 cells was greatly inhibited by c9, t11-CLA. Howerer, the 100 μM and 200 μM concentrations of c9, t11-CLA, cell proliferation were significantly inhibited. The inhibitory frequency of various c9, t11-CLA concentrations were 27.18%,

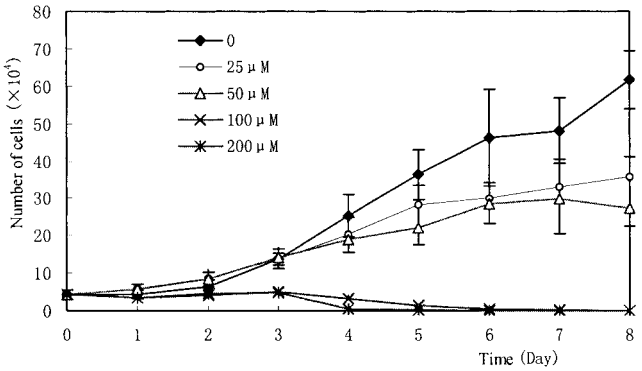

Fig. 1 Growth curve of MCF-7 cells cultured with various concentrations of c9, t11-CLA. Each data point represents the Mean±SEM. N=3

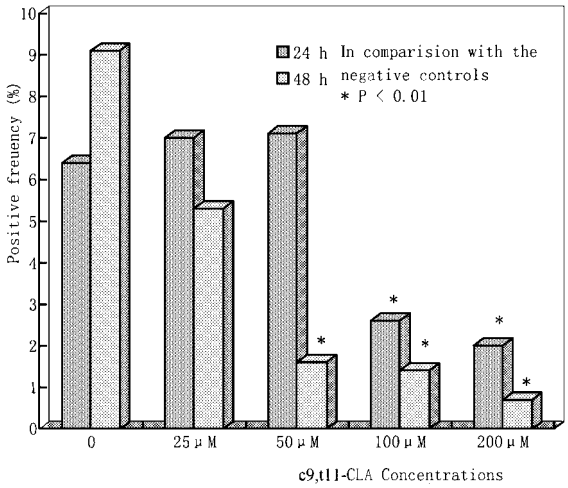

Fig. 2 Expression of PCNA in MCF-7 cells treated with c9, t11-CLA

35.43%, 91.05%, and 92.86%, respectively. In addition, MCF-7 cells were cultured with various c9, t11-CLA concentrations, and cell inhibition and death occurred in a dose- and time-dependent manner. MCF-7 cells, from this experiment, proved to be sensitive to inhibition by c9, t11-CLA.

Effect of c9, t11-CLA on DNA synthesis

The effects of c9, t11-CLA on ^{3}H-TdR incorporation into MCF-7 cells are presented in Table 1. MCF-7 cells preincubated in media supplemented with various c9, t11-CLA concentrations (except for 25 μM, 24 h) incorporated significantly less ^{3}H-TdR than did the negative controls (P<0.05 and P<0.01). The inhibitory frequency (IF) show a dose-dependent response as the concentrations of c9, t11-CLA increased. These results suggested that the DNA synthesis on MCF-7 cells was inhibited by various c9, t11-CLA.

Analysis of flow cytometry

To investigate the influence of c9, t11-CLA on the cell cycle progression of MCF-7 cells, we determined the cell cycle distribution by flow cytometry. The results are shown in Table 2, we observed a significant portion of the cells accumulating in the G_0/G_1 phase of the cell cycle of MCF-7 cells with different c9, t11-CLA concentrations at various times, whereas the portions in the G_2/M and S phases of the cell cycle were decreased. The results suggested that c9, t11-CLA may arrest the progression of the cell cycle of MCF-7 cells.

Cell proliferation

PCNA is related to cell proliferating cycle and plays a role in both the replication and repair of DNA. To determine the effect of c9, t11-CLA on the cell proliferation of MCF-7 cells, we investigated the expression of PCNA using immunocytochemistry. The results as shown in Fig. 2, the expression of the frequency of PCNA (Fig. 3.1) on MCF-7 cells gradually decreased after MCF-7 cells were incubated with different concentrations of c9, t11-CLA for various times. Moreover, PCNA in MCF-7 cells was expressed significantly less than in the negative controls (P<0.01). The expression frequency of PCNA displayed a dose-dependent relations as the concentrations of c9, t11-CLA increased.

Expressions of cyclin A, B_1 and D_1 and $p16^{ink4a}$, $p21^{waf1}$

We detected the expression of cyclins (cyclin A, B_1, D_1) and cyclin-dependent kinase inhibitors (CDKI, $p16^{in4a}$ and $p21^{waf1}$) of

Table 1 Inhibitory effect of DNA synthesis in MCF-7 cells treated by c9, t11-CLA (n=6)

c9, t11-CLA	Quantity of ^{3}H-TdR incorporation (cpm, $\bar{X}$±S)		Inhibitory frequency (%)	
(μM)	24 h	48 h	24 h	48 h
0	3,310±786	5,123±606	—	—
25	3,090±727	4,211±162**	6.6	17.8
50	2,311±235*	3,132±420**	30.2	38.9
100	2,205±354*	2,259±682**	33.4	55.9
200	2,038±430**	1,871±617**	38.4	63.4

(Different from the negative controls: * P<0.05, ** P<0.01)

Table 2 Cell cycle analysis of MCF-7 cells induced by c9, t11-CLA at 24 h and 48 h ($\bar{X}$±S) (n=5)

C9, t11-CLA	G_0/G_1		G_2/M		S	
(μM)	24 h	48 h	24 h	48 h	24 h	48 h
0	48.69±1.97	46.60±4.59	17.80±2.57	17.93±4.91	33.48±2.17	35.38±5.60
25	45.12±5.28	55.94±10.69*	18.64±7.06	10.29±3.82**	36.46±4.63	33.76±7.58*
50	49.46±2.11	64.42±7.39**	23.36±2.36	10.33±4.53**	27.41±3.99*	23.10±9.53**
100	54.95±3.89**	74.91±4.25**	11.52±2.84*	6.49±1.57**	33.54±3.15	18.60±4.29**
200	65.47±4.00**	72.80±5.95**	11.10±5.85*	7.04±4.02**	23.41±7.15*	18.17±5.59**

(Different from the negative controls: * P<0.05, ** P<0.01)

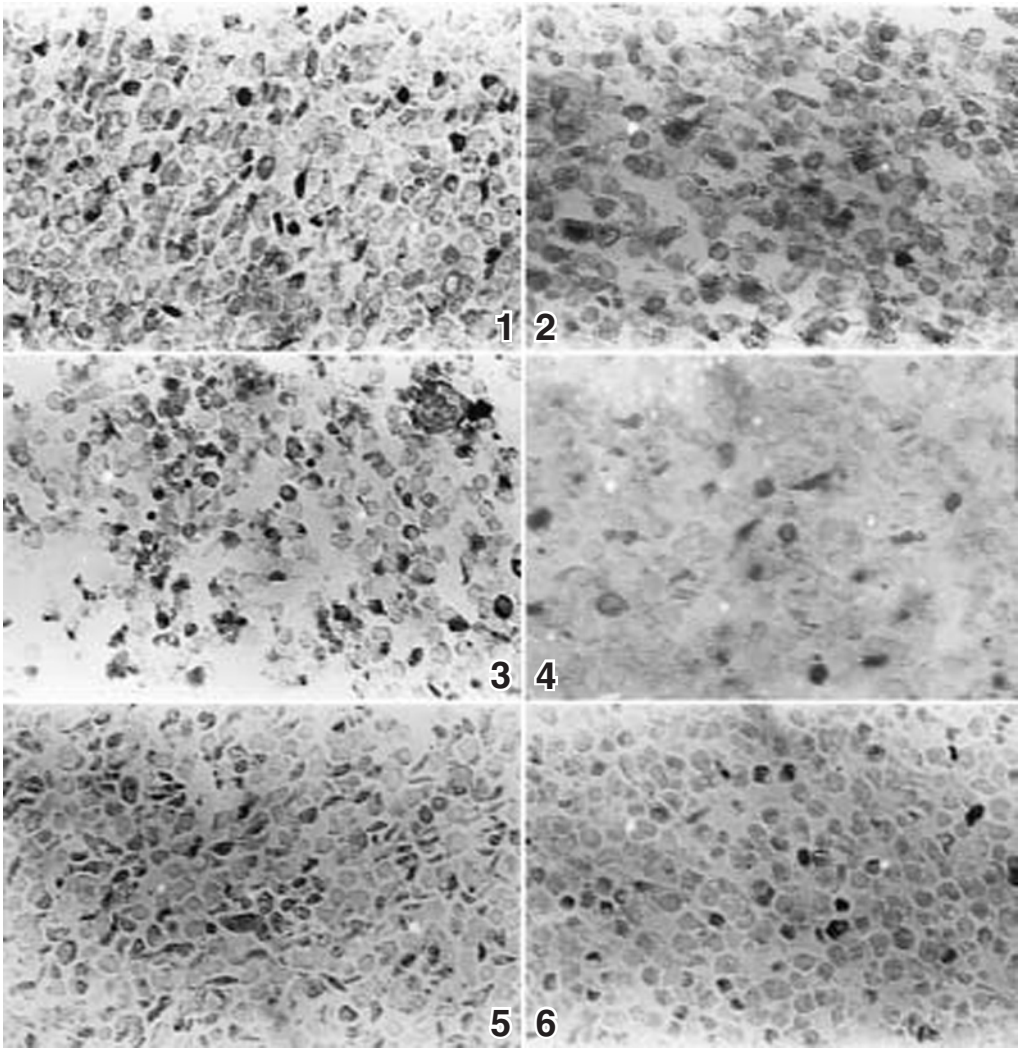

Fig. 3 MCF-7 cells immunocytochemically stained (DAB) (SP method alcohol discoloration, original magnification ×400). 1～4 are positive for PCNA, cyclin A, B_1, and D_1 in negative control. 5～6 are positive for c9, t11-CLA (200 μM, 48 h) for $p16^{inf4a}$ and $p21^{cip/waf1}$, respectively.

the cell cycle on MCF-7 cells treated with various concentrations of c9, t11-CLA by the immunocytochemical technique. The action of cyclin-dependent kinases (CDKs) the promotes cell-cycle, various cyclins bind and activate CDKs at specific times and CDKI regulates the cell cycle progression in mammalian cells. The present results suggested that the expression of the frequency of cyclin A, B_1, and D_1 (Fig. 3.2–4) on MCF-7 cells was decreased (Table 3) after MCF-7 cells were incubated with different concentrations of c9, t11-CLA for 24 h and 48 h while CDKI (Fig. 3.5–6) ($p16^{ink4a}$, and $p21^{waf1}$) increased (Table 4).

Table 3 Positive frequency (%) of cyclin A, B_1, and D_1 in MCF-7 cells treated with c9, t11-CLA

c9, t11-CLA	24 h			48 h		
(μM)	cylin A	cylin B_1	cyclin D_1	cylin A	cylin B_1	cyclin D_1
0	5.7	5.2	5.6	4.6	2.9	6.2
25	6.1	4.0*	1.8*	4.5	3.2	6.0
50	3.6*	3.8*	1.3*	1.3*	2.5	0.6*
100	1.7*	0.9*	1.0*	0.6	1.8*	0.5*
200	2.9*	0.6*	0.6*	0.5*	0.4*	0.1*

(Different from the negative controls: * P<0.01)

Discussion

CLA is a naturally occurring fatty acid in animal food products. Dietary sources of CLA include grilled beef, cheese, and related foods (13). Another source of CLA is its endogenous generation via the carbon centered free radical oxidation of linoleic acid (8). Over the past ten years, a number of animal experiments have supported the observation that CLA is an effective chemopreventive agent, and that it can inhibit carcinogenesis on different tissues at different stage induced by chemical agents (9, 11, 14). Several studies have reported that c9, t11-CLA is an effective agent to prevent carcinogenesis (15, 16) and cancer (17–

Table 4 Positive frequency (%) of $p16^{ink4a}$ and $p21^{waf1in}$ in MCF-7 cells treated by c9, t11-CLA

c9, t11-CLA	24 h		48 h	
(μM)	$p16^{ink4a}$	$p21^{waf1}$	$p16^{ink4a}$	$p21^{waf1}$
0	0.9	0.8	0.8	1.2
25	1.2	1.0	1.2	2.3
50	1.8	3.0*	2.7*	4.1*
100	2.3*	5.2*	6.6*	8.8*
200	4.0*	5.7*	7.4*	6.1*

(Different from the negative controls: * P<0.01)

19). Zhu's study (15) demonstrated that c9, t11-CLA could significantly inhibit the mice forestomach neoplasia induced by B(a)P (50 mg/kg) at postinitiation in the short term (23 weeks). The incidence of tumors in the groups of B(a)P, high dose CLA (0.1 ml/20 g) and low dose CLA (0.05 ml/20 g) was 100%, 60% and 69% respectively ($P<0.05$). Xue's research (16) also suggested that the incidence of neoplasms in mouse forestomach in the B(a)p group, 75% purity c9, t11-CLA group, 98% purity c9, t11-CLA group and 98% purity t10, c12-CLA group was 100.0%, 75.0%, 69.2%, and 53.8%, respectively. It may inhibit the mitogen activated protein kinase (MAPK) pathway to reduce neoplasms. The findings from our research group suggests that c9, t11-CLA could inhibit cell proliferation of cancer cells i.e. SGC-7901 cells (17) and MCF-7 cells (18, 19) and induced cancer cell (SGC-7901) apoptosis (20) which was produced via less expression of mutant type p53 protein possibly because of a cell cycle block.

As shown in Fig. 1, c9, t11-CLA reduced the proliferative activity of MCF-7 cells and the inhibitory frequency was between 27.18% and 92.86% in comparison with the negative controls at various concentrations of c9, t11-CLA at 8 days. The mechanism of MCF-7 cell growth inhibition by c9, t11-CLA is not yet known. However, we discovered that MCF-7 cells supplemented with c9, t11-CLA incorporated significantly less [^{3}H] thymidine than negative controls (shown in table 1): The inhibitory frequency, from 6.6% to 38.4% after incubating with c9, t11-CLA for 24 h and from 17.8% to 63.4% for 48 h, displayed a dose-dependent relation. Determining the progression of the cell cycle distribution by flow cytometry, the results showed that c9, t11-CLA may arrest the cell cycle of MCF-7 cells. Together, these results suggested that further investigations are required to clarify the mechanism of the arresting cell cycle induced by c9, t11-CLA.

In the meantime, we investigated further the expression of PCNA and protein from the cell cycle such as cyclins and cyclin-dependent kinase inhibitors (CDKI) in MCF-7 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. PCNA is an essential component of the DNA replication machinery, acting as the processivity factors for polymerases δ and ε. In addition to its role in replication, PCNA is not only required for nucleotide excision repair playing a role in one pathway of base excision repair, but also binds to cell cycle regulatory proteins such as p21 and Gadd45 (21). In the present study, we discovered that the expression of PCNA in MCF-7 cells gradually decreased in comparison with negative controls with various concentrations of c9, t11-CLA (shown in Fig. 2). In other words, DNA replication lessens and result in slower cell proliferation in MCF-7 cells.

The fundamental task of the cell cycle is to ensure that DNA is faithfully replicated once during the S-phase and that identical chromosomal copies are distributed equally to two daughter cells during the M-phase. The cell cycle is a complex process, regulated by many factors. In all, there are three parts: cyclins (A, B, D, E … H); cyclin-dependent kinase (CDK, including CDK_1～CDK_7); and CDK inhibitor (CDKI, including p16 family and p21 family). They are balanced through interactions. Uncontrolled cell proliferation is the hallmark of cancer, and tumor cells have typically acquired damage to genes that directly regulates their cell cycles. Using the immunocytochemical technique for detecting expressions of cyclins and CDKI, we have shown that the expressions of cyclin A, B_1, D_1 were reduced compared with negative control group on MCF-7 cells treated by various concentrations of c9, t11-CLA while the expression of CDKI ($p16^{in4a}$ and $p21^{waf1}$) was increased. Successive action of CDKs promotes cell-cycle progression in mammalian cells. Various cyclins bind and activate CDKs at specific times during the cell cycle.

Mammalian cyclin A activates CDK_2 (22) in the S-phase and CDK_1 (Cdc_2) in the G_2- 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 the G_1-phase increasing through the S- and G_2-phase before the protein is degraded in the M-phase. The cell cycle-dependent expression of cyclin B_1 is critical for the proper timing of a cell's entry into mitosis which is dependent both upon the binding of CDK_1 to cyclin B_1, in addition to a series of phosphorylation and dephosphorylation events. The cyclin B_1 protein accumulates during interphase and peaks at the G_2-M phase transition (23). One of the crucial substrates of the G_1 phase CDK, including CDK_4 in the complex with D-type cyclins (cyclin D_1, D_2 and D_3), is Rb protein (pRb), which is the product of the retinoblastoma susceptibility gene, and was the first tumor suppressor gene identified. Rb protein plays an important role in the regulation of the G_1 to S phase progression in normal cells and the function of pRb is regulated by phosphorylation.

Thus, during the G_0 and G_1 phase, Rb protein is in an un- or underphosphorylated state. Different stage of Rb binds to E_2F family transcription factors. Cylin Ds/CDK_4 becomes activated around the mid-G_1 phase, resulting in the accumulation of increasingly phosphorylated, inactive pRb forms. This causes the release of E_2F family transcription factors which induce the expression of S-phase genes by positive regulation through E_2F-binding sites (see Fig. 4) (24). It is also known that abrogation of the functions of Cycin A prevents entry into the S phase. Rb protein remains in the hyperphosphorylated inactive state until the end of the M-phase after entry into the S phase, after which both cylin A/CDK_2 and Cycin A, B_1/Cdc_2 (25) are thought to catalyze the phosphorylation reaction. $p16^{ink4a}$ is the founder member of a family of proteins with the ability to inhibit CDK_4 and the CDK_4-related kinase CDK_6. The INK4 family is composed of four members in mammalian organisms: $p16^{ink4a}$, $p15^{ink4b}$, $p18^{ink4c}$, and $p19^{ink4d}$. The four mammalian INK4 proteins have similar biochemical properties: all of them bind to CDK_4 and CDK_6 and inhibit the kinase activity of the $CDK_{4\text{-}6}$/Cylin D complexes (see Fig. 4) (26).

The INK4 inhibitors cause G_1 arrest suggesting that the phosohorylation of pRb on residues specific for CDK_4 (and

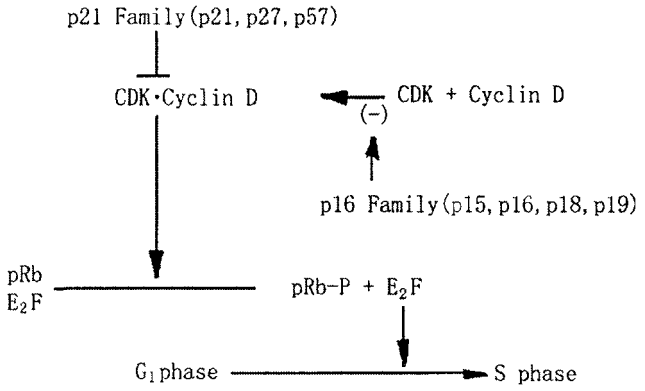

Fig. 4 The relation between CDKI (p16 and p21) and Cyclins in G_1/S transition

possibly CDK_6) is critical for G_1/S progression. While $p21^{CIP1/waf1}$ 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 Fig. 4) (26). In addition to its role as a CDKI, $p21^{Cip/waf1}$ has been shown to block DNA replication by direct interaction with the PCNA mentioned above. However, $p21^{CIP1/waf1}$ does not inhibit the PCNA-dependent nucleotide-excision repair of DNA. Indeed, DNA damage leads to an increase in the level of p53, result in p21-mediated cell cycle arrest in the G_1 phase, which persists until DNA repair is completed (27). Thus, it was proposed that $p21^{Cip/waf1}$ plays an important role under such conditions such as terminal differentiation and cell senescence.

In conclusion, c9, t11-CLA may inhibit cell growth and proliferation by a decrease in the expressions of cyclin A, B_1 and D_1 and an increase in that of CDKI ($p16^{ink4a}$ and $p21^{Cip1/waf1}$) in MCF-7 cells in comparison with the negative controls. This result suggested that the inhibition effect of c9, t11-CLA in MCF-7 cell proliferation is related to the cell cycle. The whole mechanism of action of c9, t11-CLA in the MCF-7 cell cycle, such as Rb protein and CDK, requires clarification in further studies.

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