Trans-10, cis-12, Not cis-9, trans-11, Conjugated Linoleic Acid Inhibits G1-S Progression in HT-29 Human Colon Cancer Cells

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ABSTRACT Commercial preparations of conjugated linoleic acid (CLA) contain both positional and geometric isomers of octadecadienoic acid, with cis-9,trans-11 CLA (c9t11) and trans-10,cis-12 CLA (t10c12) as the principal isomers. We showed previously that CLA reduced the incidence of colon tumors in rats treated with 1,2-dimethylhydrazine. In addition, our previous in vitro studies showed that t10c12 inhibited the growth of HT-29 and Caco-2 human colon cancer cells, whereas c9t11 had no effect on cell growth. In the present study, to examine the effects of the CLA isomers on cell cycle and cell cycle regulatory proteins, we treated HT-29 cells with various concentrations (0–4 μmol/L) of the individual CLA isomers. A DNA flow cytometric analysis revealed that t10c12 induced a G1 arrest, whereas c9t11 had no effect on the cell cycle. Western blot analysis of total cell lysates revealed no alteration in the protein expression of cyclin A, cyclin D, cyclin E, cyclin-dependent kinase (CDK) 2, or CDK4 due to t10c12 treatment. However, t10c12 substantially increased the protein expression and mRNA accumulation of the CDK inhibitor p21cip1/waf1. The t10c12 isomer increased the association of p21cip1/waf1 with CDK2 and proliferating cell nuclear antigen, but decreased the levels of phosphorylated retinoblastoma protein (Rb), with an increase in the levels of hypophosphorylated Rb protein. An in vitro kinase assay using histone H1 as a substrate showed that the activities of CDK2 were significantly decreased by t10c12. These results indicate that t10c12 exerts its growth inhibitory effects in colon cells through the induction of G1 cell cycle arrest. The induction of p21cip1/waf1 may be one of the mechanisms by which t10c12 inhibits cell cycle progression in HT-29 cells.


KEY WORDS: • conjugated linoleic acid • cell cycle • cyclin-dependent kinase • retinoblastoma protein • p21cip1/waf1

Colon cancer, next to lung cancer for men and breast cancer for women, is the second-most frequent cancer in Europe and North America (1), with the incidence of this disease also rapidly increasing in Oriental countries. Despite substantial information on the mechanisms of carcinogenesis, the survival of patients with advanced solid tumors has not increased considerably over the past 30 y (2). Recently, dietary compounds that have potential for cancer treatment have been under intensive investigation.

Conjugated linoleic acid (CLA)3 denotes a series of positional and geometric isomers of octadecadienoic acid; 2 of these [cis-9,trans-11 CLA (c9t11) and trans-10,cis-12 CLA (t10c12)] are reported to possess biological activity (3). CLA was shown to elicit cancer protection in a variety of experimental carcinogenesis models, including that of colon cancer. Much of this research involved feeding animals mixtures of CLA isomers, containing predominantly c9t11 and t10c12 in approximately equal amounts, with other CLA isomers at much lower levels [reviewed in (4)]. Evidence exists that the various biological effects of CLA are attributable to the separate actions of the c9t11 and t10c12 isomers (3), even though some effects are likely to be induced and/or enhanced synergistically by these isomers. However, the molecular mechanisms of the antitumor activity of CLA isomers remain to be elucidated.

One of the most common events required for human cancer development is deregulation of the cell cycle mechanism (5,6). The mammalian cell cycle is divided into 4 separate phases, referred to as the G1, S, G2, and M phases. During the G1 phase, cells respond to extracellular signals by either advancing toward another division or withdrawing from the cycle into a resting phase.
state (G0) (7–9). The mammalian cell cycle progression is controlled by the sequential activation and inactivation of several cyclin-dependent kinases (CDK) (10). CDK 2 and 4/6, in association with cyclins D, E, and A, sequentially phosphorylate the retinoblastoma protein (Rb), and regulate the G1→S phase transition and progression through the S phase (11). Cyclin-dependent kinase inhibitors (CKI), which bind and inhibit the activity of CDK, play crucial roles in inhibiting cell proliferation. p21cip/waf1 is the founding member of the CIP/KIP family of CKI, which also includes p27kip1 and p57kip2 (12,13). In addition to its ability to inhibit CDK, p21cip/waf1 can also bind and inhibit the activity of proliferating cell nuclear antigen (PCNA) (14,15). Because autonomous cell proliferation is a characteristic of cancer cells, cell cycle regulatory proteins are promising targets for cancer therapy.

We reported previously that dietary CLA reduces tumor incidence in the colon of 1,2-dimethylhydrazine-treated rats (16). In addition, our in vitro study showed that CLA inhibits the G1→S cell cycle progression in HT-29 cells by inducing p21cip/waf1 (17). We also observed that t10c12 inhibits the growth of HT-29 and Caco-2 human colon cancer cells, whereas e9t11 had no effect on the growth (18,19). The present study examined the effect of CLA isomers on the cell cycle progression of HT-29 cells.

MATERIALS AND METHODS

Materials. We purchased reagents from the following suppliers: e9t11 and t10c12 (Cayman Chemical); anti-β-actin, essentially fatty acid-free bovine serum albumin (BSA), ascorbic acid, α-tocopherol phosphate, and transferrin (Sigma Chemical); selenium and DMEM/Ham’s F-12 nutrient mixture (DMEM/F-12; Gibco BRL); horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG (Amersham); [γ-32P]ATP (NEN-Life Sciences); anti-phospho-Rb (Ser807/811; Cell Signaling); anti-cyclin D1 antibody (Neomarkers); antibodies against p27kip1 (c-19), p21cip/waf1 (c-19), cyclin A (c-19), cyclin E (M-20), CDK2 (M-2), CDK4 (c-22), Rb (c-15), and PCNA (PC10) (Santa Cruz Biotechnology).

Cell culture. The HT-29 cell line was obtained from the American Type Culture Collection and maintained in DMEM/F12, containing 100 mL/L fetal bovine serum (FBS) with 100,000 U/L penicillin and 100 mg/mL streptomycin. We used HT-29 cells between passages 137 and 148 in these studies. To examine the effect of the CLA isomers, we plated the cells with DMEM/F-12 containing 10% FBS. Before the CLA treatment, we rinsed the cell monolayers and serum-starved them for 24 h, with DMEM/F-12 supplemented with 5 mg/L transferrin, 1 g/L BSA, 5 μg/mL selenium, 50 μg/mL ascorbic acid, and 20 μg/mL α-tocopherol phosphate (serum-free medium). After serum-starvation, we replenished the monolayers with fresh serum-free medium, with or without the indicated concentrations of the individual CLA isomers. We made the CLA isomer complexes with essentially fatty acid-free BSA, with a molar ratio of CLA isomer to BSA of 4:1 (20). The medium was changed every 2 d.

Flow cytometric analysis of cell cycle distribution. We plated the cells in 24-well plate, at 50,000 cells/well, in DMEM/F12 containing 10% FBS. We serum-starved and then treated the cells with the indicated concentrations of the CLA isomers, as described above. Cells were separated by trypsin-EDTA, treated with RNase, and the cellular DNA was then stained with propidium iodide, as previously described (17). The percentages of cells in the G1, S, and G2/M phases of the cell cycle were analyzed by flow cytometry. The proportion of nuclei in each phase of the cell cycle was determined using the Modfit version 1.2 software (Becton Dickinson).

[^H]Thymidine incorporation. To determine [^H]thymidine incorporation, we plated HT-29 cells onto 96-well plates at a density of 6000 cells/well, serum starved and treated with CLA isomers for 57 h as described above. We then added 0.5 μCi [^H]thymidine to each well, and the incubation continued for an additional 15 h at 37°C.

[^H]Thymidine incorporation into the DNA of HT-29 cells was determined, as described earlier (19). The viable cell number was determined in duplicate wells, using the MTT assay, as described previously (21).

Immunoprecipitation and Western blot analyses. The cells were lysed in ice-cold lysis buffer, as previously described (22), and the protein content was determined using the biocinchonic acid protein assay (Pierce). Cell lysates (0.75 mg protein) were precleared with 1 μL of normal rabbit IgG and 50 μL of protein A-Sepharose bead slurry (Amersham) by incubation on an end-over-end mixer for 1 h at 4°C, and then centrifugation at 14,000 × g for 10 min at 4°C. The precleared lysates were incubated with 5 μg of an anti-CDK2 or anti-PCNA antibody for 1 h at 4°C. Protein A-Sepharose was added, and the mixture was incubated for a further 1 h at 4°C; the beads were then washed 4 times with lysis buffer by centrifuging at 600 × g for 5 min at 4°C. Total cell lysates (50 μg protein) or immunoprecipitated proteins were resolved on a SDS-PAGE (4–20% or 10–28%) and transferred onto a polyvinylidene fluoride membrane (Millipore). The blots were blocked for 1 h with 5% skimmed milk dissolved in 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween 20, and then incubated for 1 h with anti-p21cip/waf1 (1:1000), p27kip1 (1:1000), anti-cyclin D1 (1:200), anti-cyclin A (1:1000), anti-cyclin E (1:1000), anti-CDK2 (1:1000), anti-CDK4 (1:1000), anti-phospho-Rb (1:1000), anti-Rb (1:1000), anti-PCNA (1:1000), or anti-β-actin (1:2000) antibody. The blots were then incubated with anti-mouse or rabbit HRP-conjugated antibody. Signals were detected based on an enhanced chemiluminescence method using the SuperSignal® West Dura Extended Duration Substrate (Pierce). Densitometric analysis was carried out using the Bio-profile Bio-ID application (Vilber-Lourmat). Expression levels were normalized to β-actin, with the control (0 μmol/L CLA isomer) level set at 100%.

Reverse transcription-PCR. Total RNA was isolated using Tri reagent (Sigma) and cDNA synthesized using 2 μg of total RNA and SuperScript™ II reverse transcriptase (Invitrogen), as described previously (22). Amplification of cDNA was performed, as previously described (17). For each combination of primers, the kinetics of the PCR amplification were studied, the number of cycles corresponding to the plateau determined, and PCR performed within an exponential range. PCR products were separated on a 2% agarose gel and stained with ethidium bromide. Bands corresponding to each specific PCR product were quantified by densitometric scanning of the exposed film using the Bio-profile Bio-ID application (Vilber-Lourmat). The mRNA levels were normalized to β-actin, with the control (0 μmol/L CLA isomer) level set at 100%.

CDK2 activity: in vitro kinase assay. Cell lysates (0.75 mg protein) were immunoprecipitated with polyclonal antibody against CDK2 and protein A-Sepharose, and the immune complex washed with 20 mmol/L Tris-HCl, pH 7.5, and 4 mmol/L MgCl2 (kinase buffer). After being washed, the beads were incubated with 15 μL of kinase buffer, with 2 μg of histone H1 (Roche) and 3 μCi of [γ-32P]ATP, at 37°C for 30 min. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The 32P-labeled histone H1 was resolved on a SDS-PAGE, and the gel dried and subjected to autoradiography. Signals were quantified by densitometric scanning of the exposed film.

Statistical analysis. The results are expressed as means ± SEM, and were further analyzed by ANOVA. Differences among treatment groups were analyzed using Duncan’s multiple range test, utilizing the SAS system for Windows V8.12 (SAS Institute). Differences were considered significant at P < 0.05.

RESULTS

T10c12, not e9t11, induces GI arrest. We first determined which of the CLA isomers (t10c12 or e9t11) regulates the cell cycle progression of HT-29 cells. In the presence of t10c12, we observed a dose-dependent increase in the percentage of cells in the G1 phase; this accumulation was accompanied by a corresponding reduction in the percentages of cells in the S and G2/M phases (Table 1). However, e9t11
did not affect the cell cycle transition (data not shown). [3H]Thymidine incorporation into the DNA of HT-29 cells was dose dependently decreased in cells treated with 10c12 (Table 1), whereas c9t11 had no effect on DNA synthesis (data not shown).

**T10c12, not c9t11, increases p21CIP1/WAF1 levels.** Western blot analysis of total cell lysates revealed that neither c9t11 nor t10c12 had an effect on the protein levels of either CDK2 or CDK4 (data not shown). The levels of cyclins A, D1, and E remained unaltered in HT-29 cells upon treatment with the CLA isomers (data not shown). T10c12 markedly increased the expression of p21CIP1/WAF1 in a dose-dependent manner, with a 1400% increase in p21CIP1/WAF1 protein levels after the addition of 4 μmol/L t10c12 (Fig. 1A), whereas c9t11 had no effect (Fig. 1B). Neither c9t11 nor t10c12 altered the protein expression of p27KIP1 (Fig. 1A and B). To determine whether t10c12 regulates the expression of p21CIP1/WAF1 at the RNA level, we performed RT-PCR analyses. Treatment of HT-29 cells with increasing concentrations of t10c12 increased p21CIP1/WAF1 mRNA levels in a concentration-dependent manner, with a 950% increase in p21CIP1/WAF1 transcripts after the addition of 4 μmol/L t10c12 (Fig. 1C).

**T10c12 decreases CDK2 activity.** The activity of CDK2 decreased in a dose-dependent manner after t10c12 treatment. The decrease in CDK2 activity occurred in concert with enhanced binding of p21CIP1/WAF1 (Fig. 2). Therefore, p21CIP1/WAF1, which was increased after the treatment of HT-29 cells with t10c12, binds to CDK2 and inhibits its activity.

**T10c12, not c9t11, decreases hyperphosphorylated Rb but increases hypophosphorylated Rb.** Western blot analysis of total cell lysates with phospho-Rb antibody revealed a decrease in the phosphorylated Rb levels, in a t10c12 dose-dependent manner (Fig. 3). When the immunoblot was probed using the total Rb antibody, 2 bands were detected; hyperphosphorylated and hypophosphorylated Rb. A t10c12 dose-dependent decrease in the intensity of the upper band (hyperphosphorylated Rb) was detected, whereas the intensity of the lower band (hypophosphorylated Rb) was increased in cells treated with 4 μmol/L t10c12. However, c9t11 did not affect either the hyperphosphorylated or hypophosphorylated Rb levels (Fig. 3).

**T10c12 increases the levels of p21CIP1/WAF1 associated with PCNA.** Western blot analysis of total cell lysates showed that t10c12 did not change the expression of PCNA protein (Fig. 4). To determine whether t10c12 increases the levels of p21CIP1/WAF1 associated with PCNA, immunoprecipitation was performed with the anti-PCNA antibody, followed by immunoblotting with the anti-p21CIP1/WAF1 antibody. Consistent with the results from Western blot analysis, the immunoprecipitated PCNA was not affected by t10c12 treatment. However, the levels of p21CIP1/WAF1 associated with PCNA were significantly increased, in a concentration-dependent manner.

**DISCUSSION**

A deregulation of the cell cycle checkpoints is a common abnormality observed in human cancers. Most human cancers experience CDK hyperactivation, which leads to the inactivation of the Rb pathway. Accordingly, new CDK inhibitors are being developed for cancer therapy (23). CLA is currently under intensive investigation due to its health-promoting potential; in this study, we emphasize the cancer treatment potential of one isomer of CLA. A number of investigators showed that CLA inhibits carcinogenesis in experimental...
Phosphorylation of Rb proteins impairs binding to the E2F transcriptional regulators, the E2F proteins (7,40,41). Phosphorylation at or near the restriction check-point of G0-S phase coinciding with the S phase. Additional work is warranted to identify the nature of the molecules required for t10c12 to induce p21CIP1/WAF1 gene expression.

CDK phosphorylate members of the Rb protein family, with the progression from the G1-S phase coinciding with the phosphorylation and consequent inactivation of Rb proteins [reviewed in (37)]. Cyclin D-dependent kinases initiate Rb phosphorylation and consequent inactivation of Rb proteins (7,38,39). In quiescent cells, hypophosphorylated Rb associates with a family of transcriptional regulators, the E2F proteins (7,40,41). Phosphorylation of Rb proteins impairs binding to the E2F (19,26) and ErbB3 signaling in HT-29 cells (18), whereas c9t11 had no effect.

Normal concentrations of CLA in human sera were reported to be in the 7–70 μmol/L range, whereas levels > 100 μmol/L were detected in chronic alcoholics and patients with liver disease (27–29). Concentrations up to 5 times that found in normal serum were reached in humans after supplementation with 2.1 g CLA for 45 d (30). The main food source of CLA in the Western diet is meat and dairy products derived from ruminant animals with ratios of c9t11 to t10c12 of 30–70:1. However, in most of animal and human studies, commercial preparations of CLA are used as a 1:1 mixture of the 2 isomers [reviewed in (31)]. Therefore, the concentrations of the CLA isomers used in the present study are physiologically achievable if CLA-rich natural products and commercially prepared CLA are consumed.

In the present study, we showed that t10c12 treatment leads to increased expression of the CDK inhibitor p21CIP1/WAF1. Transcription of the p21 gene is regulated by both p53-dependent and independent mechanisms (32). In HT-29 cells, t10c12 induces p21 mRNA levels in a p53-independent manner because these cells lack functional p53 (33). Induction of p21CIP1/WAF1 can lead to G1 arrest by inhibition of the cyclin D1, E1, and A1-dependent kinases (34,35). Overexpression of p21CIP1/WAF1 is reported to inhibit the proliferation of mammalian cells (35,36). Our data indicate that physiological levels of t10c12 are capable of inducing sufficient levels of p21CIP1/WAF1 to inhibit both CDK activity and progression form the G1→S phase. Additional work is warranted to identify the nature of the molecules required for t10c12 to induce p21CIP1/WAF1 gene expression.

CDK phosphorylate members of the Rb protein family, with the progression from the G1-S phase coinciding with the phosphorylation and consequent inactivation of Rb proteins [reviewed in (37)]. Cyclin D-dependent kinases initiate Rb phosphorylation at or near the restriction check-point of G0-G1, after which cyclin E-CDK2 becomes active, completing this process by phosphorylating Rb on additional sites (7,38,39). In quiescent cells, hypophosphorylated Rb associates with a family of transcriptional regulators, the E2F proteins (7,40,41). Phosphorylation of Rb proteins impairs binding to the E2F
The present study demonstrated that t10c12 upregulates the level of p21CIP1/WAF1 and its interaction with PCNA, which may also contribute to the observed decreased DNA synthesis.

We observed previously that in addition to inducing growth arrest, t10c12 treatment also induced apoptosis of Caco-2 human colon carcinoma cells (19,47). Although the expression of p21CIP1/WAF1 often acts as an inhibitor of apoptosis [reviewed in (32)], it was also shown to increase apoptosis in some systems (48–53). Several groups also reported that disruption of p21CIP1/WAF1 expression leads to decreased cell death, suggesting that this CKD inhibitor has proapoptotic roles under some conditions (54–57). Poole et al. (57) showed decreased apoptosis of colonic epithelial cells in mice deficient of p21CIP1/WAF1 after treatment with the colon carcinogen, azoxymethane. It is likely that the induction of p21CIP1/WAF1 contributes to both growth arrest and apoptosis in t10c12-treated colon cancer cells. Apoptosis provides a principal protective mechanism for disposing of damaged cells that may escape growth control and would contribute to the potential therapeutic benefits of t10c12. In conclusion, the present study showed that t10c12 induced cell cycle arrest at the G0/G1 phase. An increase in the levels of p21CIP1/WAF1 in t10c12-treated cells led to the inhibition of the CKD activity, which resulted in a decrease in phosphorylated Rb and an increase in hypophosphorylated Rb. In addition, the increased p21CIP1/WAF1 bound to PCNA may have blocked the ability of PCNA to activate DNA polymerase. Together, these effects caused an arrest in the G1→S cell cycle progression, followed by inhibition of HT-29 cell proliferation. Because CKD inhibitors are thought to hold considerable potential for the inhibitory control of cancer cell proliferation, these results suggest that increasing the t10c12 level of certain foods, and combining t10c12 intake with traditional chemotherapy, could be good approaches for treating colon cancer.

LITERATURE CITED


