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Conjugated linoleic acid inhibits Caco-2 cell growth via ERK-MAPK signaling pathway

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Abstract

Conjugated linoleic acid (CLA) is a naturally occurring compound found in dairy and beef products. In recent years, it has received considerable attention because several studies showed a lower incidence of certain cancers in animals fed CLA-supplemented diets. In vitro studies further showed growth inhibitory activity on tumor cell proliferation, the CLA being effective above all against colon cancer cells.

The aim of the present work was to investigate the growth inhibitory effect of CLA on Caco-2 cell line. Under our experimental conditions, CLA repressed Caco-2 cell proliferation, and the growth-inhibitory action increased by repeating treatments. However, in Caco-2 cells, CLA was unable to induce apoptosis, as revealed by cell-cycle analysis and Western blot studies. To determine the mechanism by which CLA inhibits cell growth, we studied its effect on extracellular-regulated kinase signaling. Conjugated linoleic acid reduced expression levels of Raf-1 and phosphorylation of ERK1/2, which was accompanied by a decrease in the expression of the downstream transcription factor c-*myc*.

Our data suggest that CLA is dependent, at least in part, on the ERK kinase pathway for its ability to inhibit the growth of Caco-2 cancer cells.

Keywords: Caco-2; CLA; Proliferation; ERK cascade

1. Introduction

Nutrition and lifestyle are considered to be important risk factors in colon cancer development, one of the most common malignancies in the Western world. An association between the incidence of colon cancer and dietary factors has been revealed by numerous epidemiological and experimental studies [1]. To this regard, the importance of dietary fatty acids and their metabolites has been well established in the control of colon cancer cell growth.

Recently, a natural occurring substance of animal origin, conjugated linoleic acid (CLA), has received considerable attention as an anticancer agent. Conjugated linoleic acid is the common denomination of a heterogeneous group of C18 fatty acids with two double bonds, formed by biohydrogenation and oxidation processes in nature [2]. It consists of a mixture of positional and geometric isomers of linoleic acid, an n-6 PUFA converted by rumen bacteria via an enzymatic isomerase reaction, and so is found primarily in ruminant meat, milk and diary products [3].

Studies in animal models have shown that feeding CLA has beneficial effects on chemically induced forestomach neoplasia and skin tumors in mice [4,5], and mammary and colon carcinogenesis in rats [6,7]. Furthermore, CLA inhibits the in vitro growth of a variety of tumor cell types [8].

As regards colon cancer, dietary CLA has been shown to inhibit colon cancer incidence in rats treated with 1,2-dimethylhydrazine (DMH) [9], and in vitro studies have shown that CLA inhibits the growth of the human colon cancer cells SW480 [10] and HT29 [11,12].

Induction of apoptosis is thought to be a major mechanism of the growth inhibitory effect of CLA, both in vivo and in vitro. In fact, feeding CLA was reported to induce apoptosis in colon tissues of chemically treated rats [9,13], and providing CLA in vitro induced apoptosis in SW480 and HT29 cells [10,12].

Some authors [14,15] have suggested that a link between apoptosis and the MAPK cascade may affect the MAPKs

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controlling cell proliferation, cell differentiation and cell death. Among the three groups of MAPK cascade, the *ras*/raf-1/MEK/ERK pathway is important for cell proliferation [16]. Activation of this pathway involves a cascade of phosphorylation events initiated by the stimulation of Ras and ended with MAPK kinases (MEK1/2)-mediated dual phosphorylation of ERK1/2. ERKs play a key role in a variety of human carcinomas [17] and are up-regulated in human colorectal cancer and colon tumors in animal models [18]. Suppression of the MAPK pathway by specific inhibitors was additionally shown to induce inhibition of cell growth and apoptosis in human colon carcinoma cells [19,20].

The two ERK isoforms, ERK1 and ERK2, target transcription factors such as c-*myc* and are likely involved in carcinogenesis, as their activation is enhanced in several tumors [21,22] including colon cancers [23].

Antineoplastic compounds can exert their pharmacological activity by modulating this pathway [24–26], and inhibition of the ERK signaling has been already suggested to represent the final step of the growth inhibitory activity of CLA on HT29 colon cancer cells [27].

On the whole, the data on the anticarcinogen activity of CLA prompted us to assess the mechanisms that underlie CLA inhibition of colon cancer cell growth, focusing on the hypothesis that CLA could interfere with the ERK-mediated signaling. We investigated changes in Caco-2 cell proliferation, cell cycle and cell death, in correlation with the involvement of the ERK cascade, to gain further insight into the signal transduction pathway that may play a role in the regulation of these cellular events.

2. Materials and methods

2.1. Chemicals and reagents

Caco-2 human colorectal carcinoma cell line was a kind gift from Prof. Fiorella Biasi, Department of Clinical and Biological Sciences of the University of Torino, Italy. Conjugated linoleic acid, chemicals and culture media were purchased from Sigma (St. Louis, MO). Cell culture plasticware were from TPP (Trasadingen, Switzerland). Mouse monoclonal antibody specific to β-actin was from Sigma; rabbit polyclonal antibody specific for Raf-1 (sc-133), c-*myc* (sc-788), ERK1 (sc-94), Bak (sc-832), procaspase-3 (sc-7148), mouse monoclonal antibody specific to pospho-ERK1/2 (sc-7383), goat anti-rabbit (sc-2004) and antimouse (sc-2005) secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) detection system was from Amersham Pharmacia Biotech (Uppsala, Sweden). The protein assay kit was from Bio-Rad (Munich, Germany).

2.2. Cell culture

Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing GLUTAMAX-I and supplemented with 20% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B and 0.04 mg/ml gentamicin, at 37°C in a humidified incubator with 5% CO_2 and 95% air.

For treatments, cells were seeded at a density of 3×10^4 cells/cm^2 and grown for 24 h to allow to adhere to the substratum. The medium was then replaced with serum-free DMEM containing various concentrations of CLA and supplemented with antibiotics and antimicotics, 2 mM glutamine, 1% ITS (insulin, transferrin, sodium selenite), 1% vitamin solution and 0.4% serum bovine albumin (fatty acid free).

2.3. Cell growth and viability assay

Cells were seeded in 24-well culture plates; 1 day later, the medium was changed with DMEM containing CLA. Cell viability was assessed by the trypan blue (0.5% in NaCl) exclusion assay. Aliquots of cell suspension (100 μl) were incubated with the same volume of trypan blue for 5 min. Finally, cells were transferred to the Bürker chamber and counted by light microscope. Dead cells were defined as those stained with the dye.

2.4. Lactate dehydrogenase assay

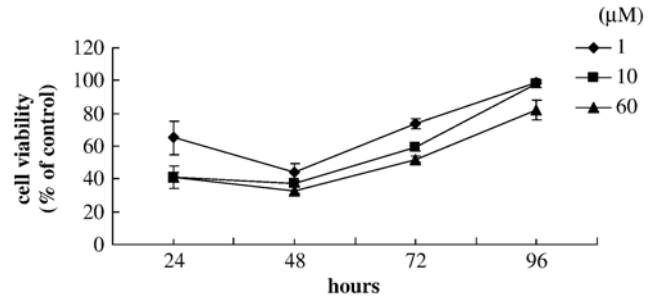
Cells were seeded in 24-well culture plates and allowed to adhere; 1 day later, cells were treated with DMEM containing CLA, and the cell supernatant was collected. The LDH activity was determined spectrophotometrically by an assay based on the oxidation of NADH and the rate of decrease in absorbance at 340 nm. The activity of LDH was calculated as nanomoles of NADH consumed per milliliter per minute.

2.5. Flow cytometric analysis

Cells were seeded in 24-well culture plates and then properly treated. After treatments, the percentage of viable, apoptotic or necrotic cells and the percentage of cells in the different phases of the cell cycle were evaluated by determining the DNA content after propidium iodide staining. Briefly, cells were washed with phosphate-buffered saline (PBS), trypsinized and centrifuged at 1000 rpm for 10 min. Pellets were fixed and resuspended in 70% ice-cold ethanol for 1 h at 4°C, then centrifuged again and incubated in PBS containing 4 mg/ml DNase-free RNase (type II-A) for 15 min at room temperature. Finally, samples were stained with 1.8 mg/ml propidium iodide for 30 min at 4°C. Data acquisition was done on a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA) and analyzed on a Macintosh computer (G3) using CellQuest software (BD Biosciences, France).

2.6. Analysis of nuclear morphology

Cells were plated on glass slides in 12-well plates; after 24 h, the medium was replaced with DMEM containing CLA. To detect nuclei, the cells were fixed with 95% ice-cold ethanol for 5 min and stained with 4′ ,6-diamidino-2-



	24 h	48 h	72 h	96 h
CLA 1 μM vs CONTROL	*	**	*	-
CLA 10 μM vs CONTROL	**	**	**	-
CLA 60 μM vs CONTROL	**	**	**	*

Fig. 1. Caco-2 cell growth curves. Cells were seeded in 24-well plates and treated with 1, 10 and 60 μM CLA for 24, 48, 72 and 96 h, respectively. Cell viability was determined by the trypan blue exclusion test. Values are means±S.D. from two independent experiments conducted in triplicate (*P<.01, **P<.001).

phenylindole (DAPI, 1 mg/ml in methanol) for 30 min at 37°C in the dark. Slides were then washed in PBS, mounted with glycerol and viewed under a fluorescence microscope equipped with a UV light filter (Dialux 20, Leitz).

2.7. Western blot analysis

Exponentially growing cells were seeded in 75 cm^2 plates and then properly treated. Cells were collected and suspended in a lysis buffer containing 20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 0.1 mM phenylmethyl–sulfonyl fluoride, 0.05% aprotinin, 0.1% Igepal.

Equal amounts of proteins (30 μg/well) were mixed with a solubilization buffer (250 mM Tris, pH 8.8, 4% sodium dodecyl sulfate (SDS), 16% glycerol, 8% 2-mercaptoethanol, 0.1% bromophenol blue) and fractionated by electrophoresis on SDS polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membranes for 2 h in a Biorad electroblotting device. The membranes were blocked with 5% milk in TBST (1 M Tris buffer saline, pH 7.4, 5 M NaCl, 0.1% Tween-20) for 1 h at room temperature. For immunodetection, the membranes were firstly incubated overnight at 4°C with primary antibody (anti-Raf-1, anti-pERK1/2, anti-c-*myc*, anti-Bak and anti-procaspase-3) and then incubated for 1 h at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized using the ECL detection system, and the band intensities were quantified by densitometric scanning. Expression of proteins was reported as a proportion of β-actin or ERK1 protein expression to control for any discrepancies in gel loading: fold change vs. control values was calculated by normalizing densitometric values obtained from the various proteins with those obtained for β-actin or for ERK1 (VersaDoc Imaging System 3000, Biorad).

2.8. Statistical analysis

Differences between means were analyzed for significance using the 1-way ANOVA test with the Bonferroni post hoc multiple comparisons, used to assess the differences between independent groups. All values were expressed as means±S.D., and differences were considered significant at P<.05.

3. Results

To determine whether CLA was effective in inhibiting Caco-2 cell growth, we incubated the cells with varying CLA concentrations (1, 10 and 60 μM) for different periods (24, 48, 72 and 96 h) in serum-free medium, and viable cells were counted by trypan blue assay. Conjugated linoleic acid significantly reduced cell growth up to 72 h, with a

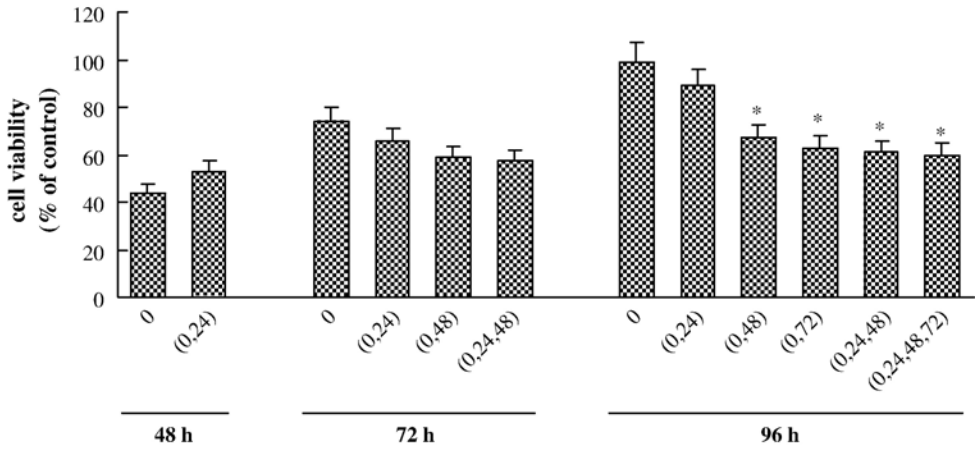


Fig. 2. Effect on cell growth of repeated doses of CLA. Caco-2 cells were seeded in 24-well plates and repeatedly incubated with 1 μM CLA for 48, 72 and 96 h with different schedules of administration: the cells received one dose (time 0), two doses (time 0 and 24, 48 or 72 h), three doses (time 0, 24 and 48 h) or four doses (time 0, 24, 48 and 72 h). Cell viability was determined at each time indicated by the trypan blue exclusion test. Values are means±S.D. from two independent experiments conducted in triplicate (*P<.01 vs. time 0).

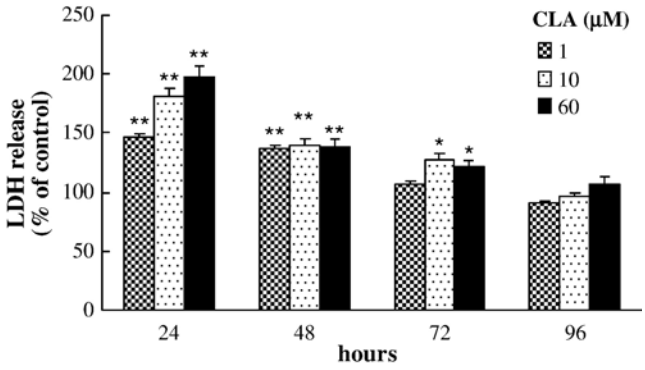


Fig. 3. Lactate dehydrogenase release. Caco-2 cells were treated with 1, 10 or 60 μM CLA for 24, 48, 72 and 96 h, respectively. LDH release is expressed as percentages of nanomoles of NADH consumed per milliliter per minute. Values are means±S.D. from two independent experiments conducted in triplicate (*$P<.01$, **$P<.001$ vs. control).

maximum decrease at 48 h (Fig. 1). Conjugated linoleic acid (60 μM) always showed the strongest inhibitory effect on cell growth as compared to lower concentrations. However, Caco-2 cell growth rate became less and less influenced by CLA when incubations were prolonged; in fact, CLA showed the lowest inhibitory effect on 96 h.

When CLA was administered to the cells for 72 h, the concentrations used were poorly effective in inhibiting cell growth, whereas when prolonging incubation up to 96 h, they became totally ineffective.

The observation that the growth inhibitory activity of CLA was abrogated by prolonging incubation prompted us to investigate whether repeated administrations to cell cultures could potentiate the inhibitory activity of CLA.

Cells were repeatedly treated with 1 μM CLA for 48, 72 and 96 h, and for any condition, cell viability was evaluated at the end of treatments (Fig. 2). The schedules of administration were as follows:

- administration of one dose (at time 0) or two doses (at time 0 and at 24 h) of CLA, viability was determined after 48 h;
- administration of one (at time 0), two (at time 0 and at 24 h, at time 0 and at 48 h) or three doses (at time 0, 24 and 48 h), viability was determined after 72 h;
- administration of one dose (at time 0), two (at time 0 and 24 h, at time 0 and 48 h), three (at time 0, 24 and 48 h) or four doses (at time 0, 24, 48 and 72 h), viability was determined after 96 h.

Dual administrations of CLA within 48 h of incubation did not influence cell growth rate, since cell viability was similar to that detected after administration of a single dose. When CLA was administered to the cells for 72 h in two or three repeated doses, it caused a higher reduction of cell growth than detected after single treatment (57–66% growth rate vs. 74%). However, a significant influence on cell growth was obtained when Caco-2 cells were repeatedly incubated with CLA over 96 h. The cell growth rate was reduced by 10% when CLA was added 24 h after the first administration, and the reduction became significantly higher in the other cases, independently of the schedule of administration. These results most likely indicate that CLA could potentiate its antiproliferative action by repeating treatments.

The cytotoxic activity of CLA was estimated by evaluating the cell release of LDH. Significant cell damage seems to occur after CLA treatment with all the concentrations used within 72 h of incubation (Fig. 3). The tendency was similar to that reported for cell growth inhibition, where 96 h of incubation with CLA had no inhibitory effect. These results likely suggest that the cell growth inhibition induced by CLA is accompanied by a cytotoxic side effect.

To determine whether CLA inhibited cell growth by interfering with cell-cycle progression, we examined its effects on cell-cycle kinetics (Fig. 4A). For the experiments, Caco-2 cells were treated with 1, 10 and 60 μM CLA for 24, 48, 72 and 96 h, respectively.

Treatment with CLA for 24 and 72 h delayed the cells in S phase and reduced their accumulation in the G2/M phase, without any effect on the fraction of cells occupying the G0/G1 phase. On the contrary, treatments for 48 and 96 h determined a slight increase in the percentage of cells accumulated in the G2/M-phase, whereas the percentage of cells occupying the G0/G1- and S-phase fraction was similar to the control percentage, suggesting that CLA effects may not be associated with modifications in cell cycle.

Flow cytometric analysis was also used to measure apoptosis. As shown in Fig. 4B, a threefold increase of apoptotic Caco-2 cells was observed in cells treated with 1 and 10 μM CLA for 48 h, and a twofold increase was found in cells treated with 10 μM CLA for 72 h, compared to untreated cells.

To better establish whether apoptotic cell death may be induced by CLA, we further examined morphologic alterations of Caco-2 cells. DAPI-stained CLA-treated cells did not show any typical apoptotic feature such as chromatin condensation or nuclear brightness, as observed by fluorescence microscope (results not shown).

In the aim to delineate whether induction of apoptosis could be excluded as a mechanism underlying the action of CLA on Caco-2 cells, we further evaluated expression levels of apoptosis-regulating proteins Bak and of procaspase-3 (Fig. 5). For these experiments, two different schedules of administration were used: (A) 1 μM CLA administered for different lengths of time and (B) different concentrations of CLA administered for 24 h. In cells treated with 1 μM CLA for different periods, the expression levels of proapoptotic protein Bak did not change (Fig. 5A), whereas Bak appears to be down-regulated by 40% after treatment with 10 μM CLA for 24 h.

On the other hand, the amount of procaspase-3 did not generally change, and it only increased after a 72-h CLA incubation.

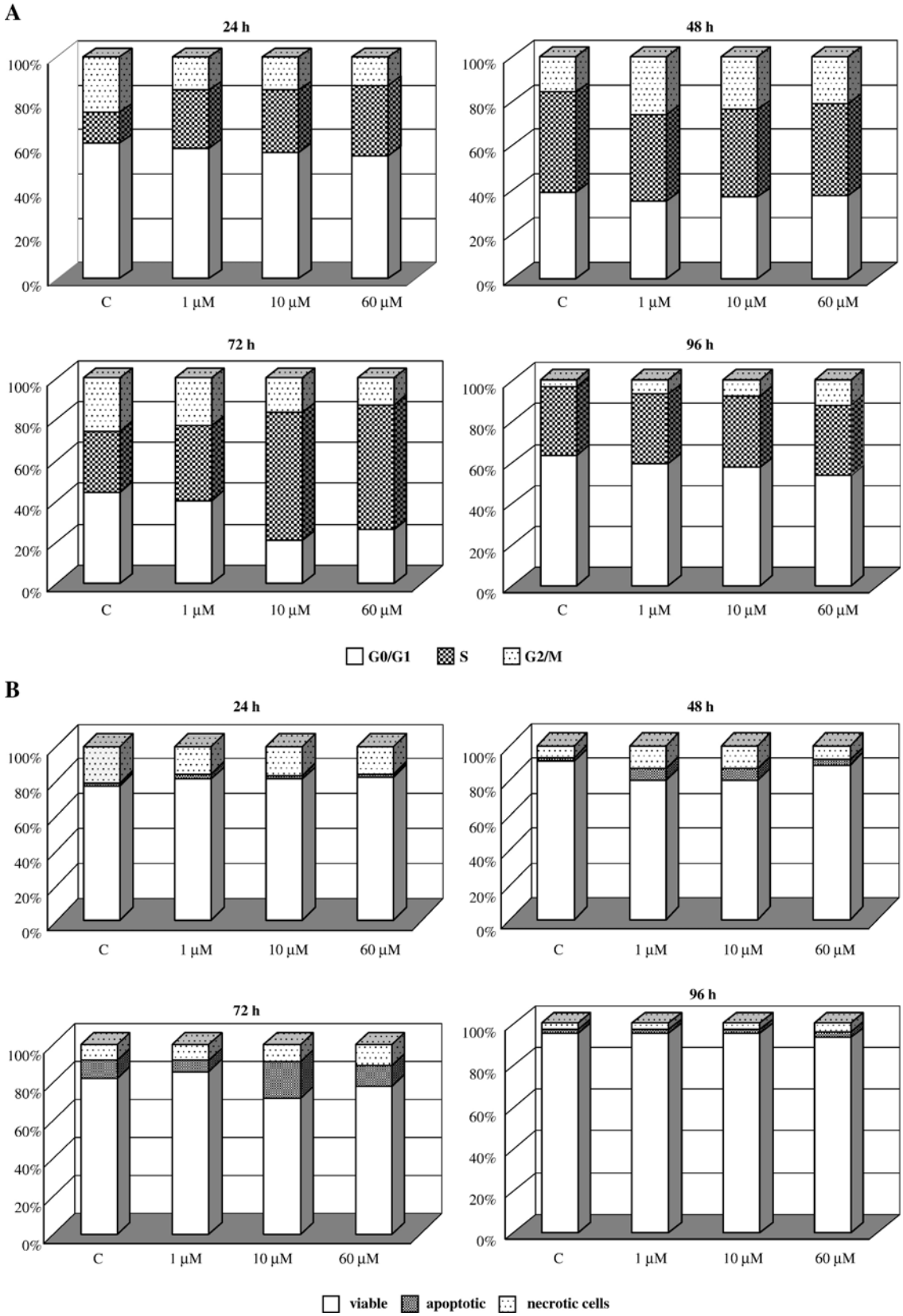


Fig. 4. Cell-cycle distribution and apoptosis. Caco-2 cells were treated with 1, 10 and 60 μM CLA for 24, 48, 72 and 96 h, respectively. Cell-cycle distribution (panel A) and apoptosis (panel B) were determined in samples stained with propidium iodide and measured by flow cytometry. Data shown here are from a representative experiment repeated three times and are expressed as percentages of total cell number with less than 10% variation.

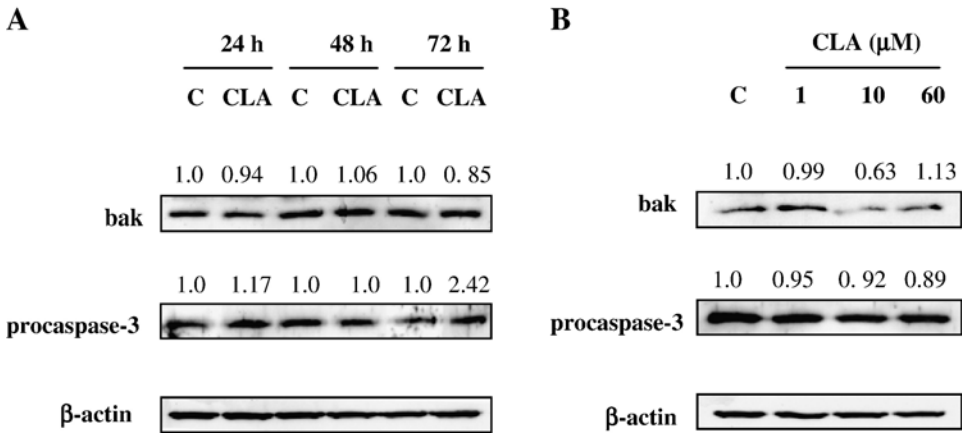


Fig. 5. Western blot analysis of Bak and procaspase-3. Caco-2 cells were treated with 1 μM CLA for 24, 48 and 72 h (panel A) or with different concentrations of CLA (1, 10 and 60 μM) for 24 h (panel B). The cell lysates were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membrane and probed with anti-Bak (A) or antiprocaspase-3 (B) antibody. Protein contents were normalized by probing the same membrane with anti-β-actin antibody.

In an attempt to explore whether CLA may affect specific signal transduction pathways, we further evaluated the effect of CLA on the ERK-MAPK cascade, a large network of signaling molecules regulating cell growth and differentiation. Immunoblotting analysis was then performed after treating the cells using the same schedule of administration described above (Fig. 6).

Conjugated linoleic acid down-regulated Raf-1 protein expression, the main reduction being observed after a 48-h incubation with 1 μM concentration.

The levels of pERK were markedly reduced by 24 h of treatment with 10 and 60 μM CLA, but the highest magnitude of reduction (80%) was detected when cells were treated for 48 h with 1 μM CLA. The reduction in the phosphorylation state of ERK-1/2 is in accordance with the results obtained from cell growth evaluation.

Inhibition of ERK most likely resulted in down-regulation of the transcription factor c-*myc* involved in the regulation of cell proliferation and apoptosis. Likewise, strong reduction occurred especially after 24 h of incubation with 10 and 60 μM CLA and after 48 h of incubation with 1 μM CLA, consistent with CLA-induced reduction of pERK1/2 proteins.

4. Discussion

Conjugated linoleic acid is a general term for the geometrical and positional isomers of octadienoic (18:2)

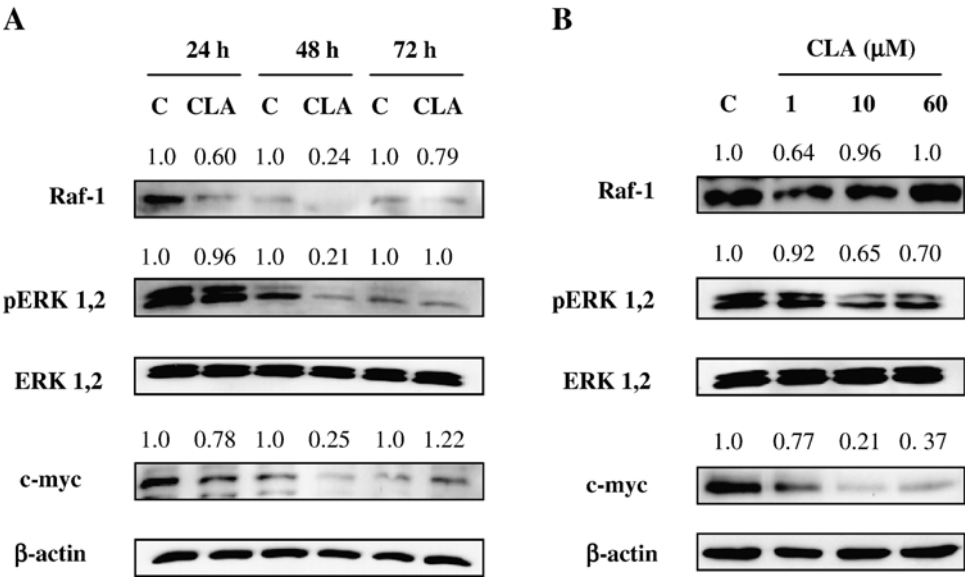


Fig. 6. Western blot analysis of Raf-1, pERK1/2 and c-*myc*. Caco-2 cells were treated with 1 μM CLA for different lengths of time (24, 48 and 72 h, panel A) or for 24 h with different concentrations (1, 10 and 60 μM, panel B) of CLA. The cell lysates were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membrane and probed with anti-Raf-1, anti-pERK1/2 and anti-c-*myc* antibody. Protein contents were normalized by probing the same membrane with anti-β-actin (for Raf-1 and c-*myc*) or with anti-ERK1 antibody (for pERK1/2).

acid, two of which (*cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA) are known to possess biological activity [28]. Conjugated linoleic acid has anticarcinogenetic properties, evidenced mainly by studies from in vivo and in vitro models of cancers. Its protective effect as antitumor food in animal models has been observed for a broad range of chemical carcinogens [3,6,13], and studies in different colon cancer cell lines demonstrated that CLA represses cell proliferation and induces apoptosis [12,10]. Among the two main isomers, the t10,c12-CLA has been shown to be an effective inhibitor of cell growth and inducer of apoptosis [29]. Conjugated linoleic acid acts through different mechanisms: it can down-regulate ErbB3 and IGF-I signaling [27,30], decrease IGF-II secretion [31,32] or induce the cyclin-dependent kinase inhibitor p21(CIP1/WAF1) [33] and stimulate the antitumorigenic protein NAG-1 [29].

However, the molecular mechanisms responsible for the antiproliferative action of CLA are not entirely understood. The present studies were thus performed to better establish the role of CLA in regulating Caco-2 human colon cancer cell growth. All concentrations of CLA were somewhat efficacious in reducing Caco-2 viable cell numbers for up to 72 h of incubation, whereas CLA did not exhibit any growth inhibitory effect when the cells were incubated over 96 h. On the other hand, when Caco-2 cells were incubated with CLA by repeat treatments, they responded by decreasing cell number more significantly as compared to single treatments. One explanation could be that CLA may disappear from the culture medium and be incorporated into the cancer cells. However, the inhibitory effect of repeated treatments of CLA on the growth of Caco-2 cells is higher than that obtained after a single administration, and these data likely indicate that repeated addition could potentiate the antiproliferative action of CLA. Our results would be taken into consideration in estimating antitumor properties of CLA, in the aim of long-term supplementation studies in cancer patients.

It is unknown whether CLA may regulate the expression of cell-cycle checkpoints. As regards Caco-2 cells, the antiproliferative activity of CLA can be associated with a delay of the cells in S phase observed for up to 72 h incubation, whereas renewal of growth activity observed after long-time administrations could be related to lack of inhibition of cell-cycle progression.

Cancer can be described as a disorder of the balance between cell proliferation and cell death, growth being determined not only by increased cell proliferation but also by decreased apoptosis [34]. Conjugated linoleic acid was previously demonstrated to induce apoptosis in colon cells [10,27]: it increased the apoptotic index in the colonic mucosa of rats injected with DMH [9] and induced caspase-dependent apoptosis in MIP-101 human colorectal carcinoma cells [11].

By contrast, in the current study, we showed that the antiproliferative effect of CLA in the Caco-2 cell line cannot be attributed to the induction of apoptosis, as no evidence of the presence of sub-G0/G1 pick in cell distribution was detected. Similarly, no changes in expression levels of Bak and of procaspase-3 occurred, confirming that CLA did not trigger apoptosis in these cancer cells. Bak is an inducer of apoptosis that mediates cytochrome *c* release from the mitochondrial intramembrane space to the cytoplasm [35], and efflux of cytochrome *c* subsequently results in cleavage and the activation of caspase cascade, a hallmark of apoptosis [36].

Proliferation of cancer cells is often associated with ERK activation [37], whereas inhibition may likely be induced through down-regulation of the ERK1/2 signal pathway [22,23]. Increasing evidence supports the idea that the CLA-induced signaling pathway is mediated through the modulation of the ERK-MAPK cascade [27], a large network of signaling molecules regulating cell growth and differentiation. Prior work from our laboratory already demonstrated that growth inhibition by CLA of the MCF-7 and MDA-MB-231 breast cancer cells involves the ERK1/2 signaling [38,39]; therefore, we performed additional experiments in the hypothesis that down-regulation of this cascade could represent one of the mechanisms by which CLA inhibits Caco-2 cancer cell growth.

Pharmacological inhibitors of the Raf/MEK/ERK pathway have been proposed as anticancer drugs [24–26]. Among them are quinoxaline dioxides, which exert antiproliferative and antiapoptotic effects in colon cancer cells [35,40], and selenomethionine, used in chemoprevention trials, which induces phosphorylation of the MAPK-ERK in association with cell-cycle arrest and growth inhibition [15].

ERK phosphorylation is known as an important step in the MAPK cascade and is implicated in mitogenic signaling [16]. Raf-1, an upstream component of the MAPK pathway, determines the amplitude of ERK activity [25], and c-*myc* is a downstream signaling targeted gene of the MAPK pathway. Therefore, the reduction in Raf-1 protein levels may likely contribute to the subsequent reduction in both pERK and c-*myc* protein amounts found in CLA-treated cells. We observed the main reduction in expression levels of these proteins after 48 h of incubation with 1 μM CLA, in accordance with results obtained in growth inhibitory assays. Different results were observed for c-*myc* protein, a transcription factor that serves key functions in cell proliferation, differentiation and apoptosis [41]. Results obtained for c-*myc* indicate that this transcription factor may represent a target for CLA and are consistent with other reports that recognize c-*myc* as a target gene for CLA [42,21,23]. The c-*myc* oncogene is a downstream target gene of the MAPK pathway and is among the most commonly overexpressed genes in human cancer [44]. Its down-regulation could reduce progression in cell cycle and influence apoptosis, and repression of the pathway ending in c-*myc* activation could represent one mechanism by which CLA exerts its biological activities.

In summary, with the current results, we can speculate that the MAPK-ERK signal transduction pathway may be targeted by CLA, consistent with previous reports suggesting that, in human colon cancers, the ERK signal transduction pathway is involved in tumor promotion and malignant progression [18]. Given the known role of MAPK in signal transduction and the regulation of cell survival and death, the present study offers a contribution to establishing whether the inhibition of MAPK signaling may represent the molecular basis underlying the preventive effects of CLA in the development of colon cancer.

Acknowledgments

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