Growth Inhibition and Apoptosis Induction by All-trans-conjugated Linolenic Acids on Human Colon Cancer Cells

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Abstract. Conjugated linolenic acids (CLN) are geometric and positional isomers of linolenic acid. Growth inhibition and apoptosis induction by α-eleostearic acid (c9,t11,t13-CLN), β-eleostearic acid (t9,t11,t13-CLN), α-calendic acid (t8,t10,c12-CLN) and β-calendic acid (t8,t10,t12-CLN) were compared. β-Eleostearic acid and β-calendic acid, which have all-trans-conjugated double bonds, exerted stronger growth inhibition and more DNA fragmentation, an indicator of apoptosis induction, in the human colon cancer cells Caco-2 than α-eleostearic acid and α-calendic acid with the cis configuration. Down-regulation of bcl-2 and up-regulation of bax mRNA by β-eleostearic acid were also greater than by α-eleostearic acid. Interestingly, the cytotoxic effects of β-eleostearic acid and β-calendic acid were not counteracted completely by α-tocopherol, whereas the cytotoxic effects of α-eleostearic and α-calendic acids were lost in the presence of α-tocopherol. These results suggest that β-eleostearic and β-calendic acids have signaling pathways different from those of α-eleostearic and α-calendic acids and exhibit high potency for reducing the cell viability of Caco-2.

The term "conjugated fatty acids" is generic for polyunsaturated fatty acids with conjugated double bonds in the molecule. Conjugated linoleic acids (CLAs) is known to have many health benefits such as anticancer (1-3), anti-obesity (4, 5) and anti-atherosclerotic effects (6, 7). Conjugated linolenic acids (CLNs), which are geometric and positional isomers of linolenic acid, are also found in high concentrations in some kinds of plant seeds. For example, α-eleostearic acid (c9,t11,t13-CLN) and α-calendic acid (t8,t10,c12-CLN) are contained in bitter gourd seed oil (BGO) and pot marigold seed oil, respectively (8). We recently demonstrated that dietary BGO, which contains α-eleostearic acid, remarkably inhibited the development of azoxymethane-induced colonic aberrant crypt foci (ACF) (9) and adenocarcinoma in F344 rats (10). Furthermore, we and others have reported that free fatty acid prepared from BGO induced apoptosis in colon cancer cells (11, 12).

On the other hand, β-eleostearic acid (t9,t11,t13-CLN) and β-calendic acid (t8,t10,t12-CLN), with all-trans-conjugated double bonds, are also known to be contained in some seed oils as minor fatty acids (8). All-trans-CLN isomers are also found in mixtures of CLNs chemosynthesized by the alkaline isomerization of linolenic acid (13). Recently, Igarashi and Miyazawa reported that β-eleostearic acid had a stronger antiproliferative effect than α-eleostearic acid (13). However, there has been no report on apoptosis induction by all-trans-CLN isomers. To characterize the anticancer effects of all-trans-CLN isomers in detail, the first study on apoptosis induction by β-eleostearic and β-calendic acids in comparison to α-eleostearic and α-calendic acids was performed here. Furthermore, it demonstrated that the effects of the antioxidant α-tocopherol on reducing the cell viability of Caco-2 differ among all-trans-CLN isomers and other isomers with the cis configuration.

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100 μg/mL streptomycin. The cell cultures were maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Cell viability assay. Caco-2 cells (2x10³ cells) were pre-incubated in 96-well plates with 100 μL medium per well for 24 h. Each CLN isomer was dissolved in ethanol and was then added into the culture medium to a final concentration up to 50 μM. The final concentration of ethanol was below 0.1% (v/v). The Caco-2 cells were incubated for 21 h to 45 h in culture medium containing CLN with/without α-tocopherol at 2.5 μM~50 μM. Then, 10 μL of WST-1 solution (14) were added to each well and the culture plate was incubated for an additional 3 h. The number of viable cells were measured at 450 nm. Viability was expressed as a percentage of the viable cells of the control culture.

Measurement of DNA fragmentation. DNA fragmentation was measured as an indicator of apoptotic cells using a commercial kit (Cell Death Detection ELISAPLUS, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. The assay is based on a quantitative sandwich enzyme immunoassay to detect the histone-associated DNA fragments produced during apoptosis. The cell culture conditions were the same as in the cell viability assay.

Extraction of total RNA. Caco-2 cells (5x10⁵ cells) were cultivated in 100-mm tissue culture dish for 24 h and each CLN isomer in ethanol was then added to the culture dish. Total RNA was extracted from the Caco-2 cells by an acidic phenol method and further purified by using an RNasea Mini Kit (Qiagen, Chatsworth, CA, USA), according to the manufacturer’s instructions.

Real-time quantitative RT-PCR analysis. Total RNA was reverse-transcribed by a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol. Then, 1 μl of cDNA solution (adequate concentration) was mixed with 1.25 μL TaqMan probe, 12.25 μL TaqMan Master Mix, 10.25 μL water and the quantitative RT-PCR reaction was performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing and extension at 60°C for 1 min. TaqMan probes, bcl-2 (Hs009999903_m1), bax (Hs00608023_m1) and β-actin (Hs00180269_m1), used in this study were purchased from Applied Biosystems.

Statistical analysis. The data are expressed as means±SD. The statistical analysis between two groups (Figure 2) was determined using the unpaired Student’s t-test. Differences with p<0.05 were considered significant. Statistical analyses between multiple groups were determined by Holm’s test (p<0.05) (Figures 3, 4, 5).

Results

Effect of CLN isomers on Caco-2 cell viability. To compare the cytotoxic effects of the CLN isomers, Caco-2 cells were incubated in culture medium containing α-eleostearic acid, β-eleostearic acid, α-calendic acid and β-calendic acid (Figure 1). As shown in Figure 2, each CLN isomer exerted a strong cytotoxic effect against the Caco-2 cells in a dose-and time-dependent manner. The β-eleostearic acid and β-calendic acid isomers, in particular, which have the all-trans configuration, showed stronger cytotoxic effects than the α-eleostearic acid and α-calendic acid isomers, which have the cis configuration, at concentrations of 3.125 μM to 12.5 μM after 24-h incubation. At 48-h incubation, β-eleostearic acid and β-calendic acid also remarkably reduced the cell viability compared to α-eleostearic acid and α-calendic acid at 3.125 μM and 6.25 μM.

Comparison of apoptosis induction in Caco-2 cells by CLN isomers. The DNA fragmentation in Caco-2 cells incubated in culture medium containing CLN isomers was measured as an indicator of apoptosis induction. All of the CLN isomers used in this study induced DNA fragmentation in Caco-2 cells in a dose-dependent manner during 48-h
incubation (Figure 3). Relative DNA fragmentation, induced by 6.25 μM and 12.5 μM β-eleostearic acid, increased to 3.5-fold and 6.9-fold the level of the control after 48 h of incubation, while DNA fragmentation by α-eleostearic acid was 1.5-fold and 4.9-fold the level of the control, respectively. β-Calendic acid also induced more DNA fragmentation in Caco-2 cells than did α-calendic acid at 6.25 μM, although the DNA fragmentation induced by 12.5 μM α-calendic acid and β-calendic acid was of the same level. Thus, β-eleostearic acid and β-calendic acid induced more DNA fragmentation than did α-eleostearic acid and α-calendic acid, respectively. This greater fragmentation corresponds to the reduction of cell viability by each CLN isomer. Furthermore, the down-regulation of bcl-2 mRNA, which is an anti-apoptotic gene, was observed in the Caco-2 cells treated with 10 μM β-eleostearic acid for 24 h (Figure 4). In addition, β-eleostearic acid up-regulated pro-apoptotic bax mRNA up to 1.4-fold in Caco-2 cells, while α-eleostearic acid did not affect bax mRNA expression. The greater DNA fragmentation and regulation of apoptosis-related genes corresponded to the reduction of cell viability by each CLN isomer.

Influence of α-tocopherol on reducing Caco-2 cell viability by CLN isomers. In previous studies, we and others have reported that the cytotoxic effect of and apoptosis induction by α-eleostearic acid are induced through intercellular lipid peroxidation (12, 15, 16). To investigate the mechanisms underlying the cytotoxic effects by all-trans-CLN isomers, Caco-2 cells were incubated with CLN isomers and α-tocopherol. When 25 μM α-tocopherol was added to the culture medium, there was no reduction of cell viability by 50 μM α-eleostearic acid, as found in previous reports (Figure 4) (12, 16). On the other hand, the cytotoxic effect of 50 μM β-eleostearic acid was observed even in the presence of 25 μM α-tocopherol, although the viability of the Caco-2 cells was partially restored, to 50% from 0.7%, by the addition of α-tocopherol (Figure 5).

Furthermore, the effect of α-tocopherol on the growth inhibition effects of CLN isomers was examined in detail (Figure 6 a). The viability of the Caco-2 cells treated with 25 μM β-eleostearic acid increased as the α-tocopherol concentration increased. However, cell viability reached a plateau at 70% and the cytotoxic effect of β-eleostearic acid was not restored completely by the addition of α-tocopherol.
The cytotoxic effect of 25 μM β-calendic acid also remained in the presence of α-tocopherol (Figure 6 b). In contrast, the cytotoxic effects induced by 25 μM β-eleostearic and β-calendic acids were lost completely by the addition of α-tocopherol (Figure 6 a, b).

**Discussion**

The results of the present study demonstrated that two all-trans-CLN isomers, β-eleostearic and β-calendic acids, have more potent cytotoxic effects and higher levels of apoptosis induction in Caco-2 cells than do α-eleostearic and α-calendic acids, CLN isomers with the cis configuration. Furthermore, β-eleostearic acid and β-calendic acid showed cytotoxic effects even in the presence of α-tocopherol, whereas α-tocopherol counteracted the cytotoxic effects of α-eleostearic acid and α-calendic acid.

CLNs are found at high concentrations in the oils of certain kinds of seeds (8). α-Eleostearic and α-calendic acids are major fatty acids of bitter gourd seed oil and pot marigold seed oil, respectively. In addition, β-eleostearic acid and β-calendic acid, which have all-trans-conjugated double bonds, are found in some kinds of seed oils (8). Moreover, all-trans-CLN isomers are produced by the alkaline isomerization of linolenic acid (13). In previous studies, we and others have reported that α-eleostearic and α-calendic acids exhibited strong cytotoxic effects on human monocyctic leukemia cells (U-937) (15) and colon cancer cells (12, 16). Recently, Igarashi et al. reported that β-eleostearic acid inhibits the growth of human tumor cell lines and that its effect is stronger than that of α-eleostearic acid (13). However, there are no detailed reports about the antiproliferative effects and apoptosis induction by all-trans-CLN isomers. In the current study, we demonstrated, for...
the first time, that two all-trans-CLN isomers, β-eleostearic acid and β-calendic acid, induced apoptosis in Caco-2 cells, and that their apoptosis activities were higher than those of α-eleostearic acid and α-calendic acid, which have the cis configuration. Furthermore, the down-regulation of bcl-2 mRNA and the up-regulation of bax mRNA in Caco-2 cells by β-eleostearic acid were greater than those by α-eleostearic acid. Bcl-2 and Bax proteins are known to function as anti-apoptotic and pro-apoptotic factors, respectively, in mitochondria (17). These results indicate that the configuration of conjugated double bonds is important with respect to the cytotoxic effect and apoptosis induction in Caco-2 cells. In particular, the all-trans-CLN isomers were more effective chemotherapeutic compounds.

The mechanism underlying the cytotoxic effect and apoptosis induction by α-eleostearic acid involves lipid peroxidation, since the antioxidant α-tocopherol diminished the growth inhibition and apoptosis induction by α-eleostearic acid on the colon cancer cell lines. In the present study, we also observed that the cytotoxic effects

Figure 4. Expression level of bcl-2 and bax mRNA in Caco-2 cells treated with α-eleostearic acid and β-eleostearic acid. Caco-2 cells were treated with 10 μM α-eleostearic acid or β-eleostearic acid for 24 h. The expression levels of bcl-2 and bax mRNA were estimated by real-time PCR. Data from three independent experiments were normalized to the β-actin mRNA level and are shown as the means±SD. The values with different letters were significantly different from each other. p<0.05.

Figure 5. Effect of α-tocopherol on antiproliferation by α-eleostearic acid and β-eleostearic acid. Caco-2 cells were treated with 50 μM CLN isomers with/without 25 μM α-tocopherol for 48 h. The cell viability was measured by the WST assay described in Materials and Methods. The data represent cell viability expressed as a percentage of the control, which was taken to be 100%. Values are means±SD, n=6. The values with different letters were significantly different from each other in the no α-tocopherol group and the 25 μM α-tocopherol group. p<0.05.

Figure 6. Comparison of antiproliferation by CLN isomers in the presence of α-tocopherol. (a) Caco-2 cells were treated with 25 μM α-eleostearic acid, β-eleostearic acid and α-tocopherol after 24 h of pre-incubation. Values are means±SD, n=6. (b) Caco-2 cells were treated with 25 μM α-calendic acid, β-calendic acid and α-tocopherol after 24 h of pre-incubation. The presented data are shown as cell numbers relative to the control. Values are means±SD, n=6.
and apoptosis induction of α-eleostearic and α-calendic acids were completely suppressed by the addition of α-tocopherol to the culture medium, as previously reported (12, 14, 15). It is noteworthy that the cytotoxic effect of β-eleostearic acid and β-calendic acid, which have all-trans-conjugated double bonds, were not counteracted completely in the presence of α-tocopherol (Figures 5, 6). These results suggest that β-eleostearic acid and β-calendic acid reduce the cell viability of Caco-2 via another pathway in addition to the pathway of lipid peroxidation. On the other hand, the cytotoxic effect of β-eleostearic acid on DLD-1, another colon cancer cell line, was lost by the addition by α-tocopherol (data not shown). Thus, the metabolic and signal transduction systems in Caco-2 cells may be important for different anticancer effects among CLN isomers. Further investigations are required for a better understanding of the specific mechanisms underlying the cytotoxic effects and apoptosis induction by β-eleostearic and β-calendic acids.

In summary, two all-trans-CLN isomers, β-eleostearic and β-calendic acids, markedly reduced the cell viability and induced apoptosis in Caco-2 cells. Their effects were stronger than those of α-eleostearic and α-calendic acids, which have the cis configuration. Furthermore, since the cytotoxic effects of β-eleostearic and β-calendic acids on Caco-2 cells were not suppressed completely by α-tocopherol, CLN isomers with the all-trans configuration are suggested to exert anticancer effects through signaling pathways different from those of the CLN isomers with the cis configuration.

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References


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