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Review

Conjugated Fatty Acids: the Relationship between Oxidative Stability and Physiological Activities

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Abstract

The conjugated linoleic acid (CLA) is now well-known among lipid scientists and nutritionists. However, there are more highly conjugated fatty acids than CLA, some of which can be prepared by alkaline isomerization of α-linolenic acid. The bioactive properties of these conjugated lipids have been studied extensively. When examined in vitro, the oxidation rate of fatty acids with conjugated unsaturation, such as conjugated α-linolenic acid including α-eleostearic acid (a natural form of conjugated α-linolenic acid), was notably faster than that of the unconjugated fatty acid (α-linolenic acid) and CLA. In cell culture study, α-eleostearic acid showed potential as novel anti-tumour agents through their ability to cause membrane lipid peroxidation and apoptosis; this was probably a result of the high susceptibility to oxidation of these conjugated fatty acids. Moreover, the inhibitory effects of α-eleostearic acid on tumour development were confirmed in vivo by using nude mice with transplanted tumour cells. Against this background, the author examines the relationship between oxidative stability and physiological activities (especially anti-tumour effect) of conjugated fatty acid.

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Introduction

Fatty acids with conjugated double bonds occur in nature (**Figure 1**). For instance, conjugated linoleic acid (CLA, 18:2), a mixture of geometrical and positional isomers of linoleic acid (9*c*12*c*-18:2), is present in ruminant fats, beef tallow and milk fat. The predominant isomer in this mixture is 9*c*11*t*-CLA. Since CLA in foodstuffs represents only about 1% of total fatty acid, vegetable oils such as safflower oil are subjected to alkaline isomerization for producing CLA-enriched oil.

Under natural conditions, seeds of some plants contain conjugated triene and tetraene fatty acids, such as α-eleostearic acid (9c11t13t-18:3), calendic acid (8t10t12c-18:3) and parinaric acid (9c11t13t15c-18:4). Furthermore, seaweeds including red and green algae contain more highly unsaturated conjugated fatty acids such as

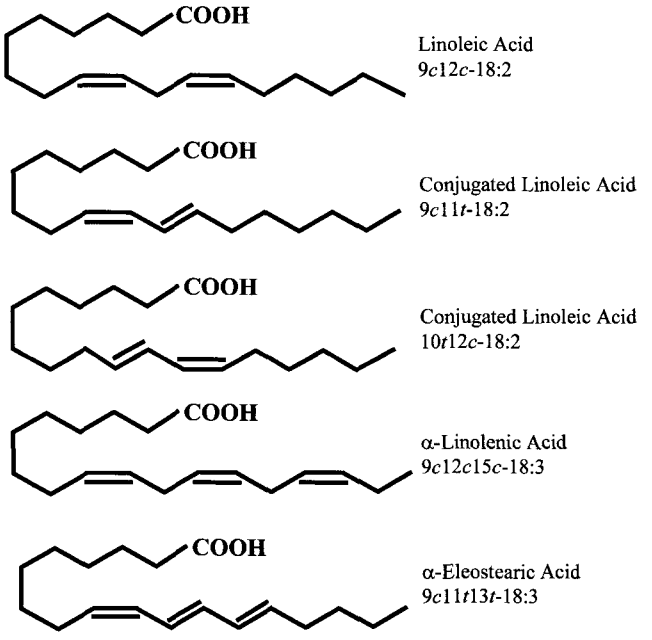

Figure 1. Chemical structures of fatty acids with and without conjugated unsaturation.

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conjugated eicosapentaenoic acid (5c7t9t14c17c-20:5), bosseopentaenoic acid (5c8c10t12t14c-20:5) and stellaheptaenoic acid (4c7c9t11t13c16c19c-22:7). In contrast to CLA, these conjugated fatty acids have received much less attention.

In this paper, I review the physiological activity of conjugated fatty acids, and discuss the possible relationship between their oxidative stability and a specific, significant physiological activity, i.e. induction of tumour selective apoptosis.

Physiological activities of conjugated fatty acids

CLA was reported first to have an anti-carcinogenic effect and, subsequently, physiological effects (prevention of atherosclerosis and regulation of lipid metabolism) (1). These physiological activities of CLA are always associated with the conjugated double bonds. The magnitude of the physiological activity varies widely among the CLA isomers; for example, 10*t*12*c*-CLA (a minor isomer in nature) shows a stronger anti-tumour and anti-obesity effect than 9*c*11*t*-CLA.

In contrast, studies have only rarely been made of the physiological functions of fatty acids with greater conjugation than CLA. This prompted us to take an interest in the bioactivity potential of such highlyconjugated fatty acids, and we anticipated greater activity than for CLA. Consequently, we prepared a series of conjugated fatty acids by alkaline isomerization of α-linolenic acid (9*c*12*c*15*c*-18:3).

Interestingly, these conjugated fatty acids showed a stronger cytocidal effect on human cultured tumour cells than found for CLA, with the proposed mechanism involving apoptosis induction through membrane lipid peroxidation (2). Moreover, our *in vivo* studies showed that the anti-tumour effects of α-eleostearic acid were greater than that of CLA (2-5). They also demonstrated that tumour selective apoptosis induction was closely involved with tumour lipid peroxidation caused by the intake of conjugated fatty acids (α-eleostearic acid). These results suggested that the bioactivities of conjugated fatty acids relate to their oxidative stability (6). Further details of these findings (2-8) are discussed here.

Oxidative stability of conjugated fatty acids in vitro

The oxidative stabilities of α-eleostearic acid, conjugated α-linolenic acid and CLA were investigated and compared (6) (**Figure 2**). On the basis

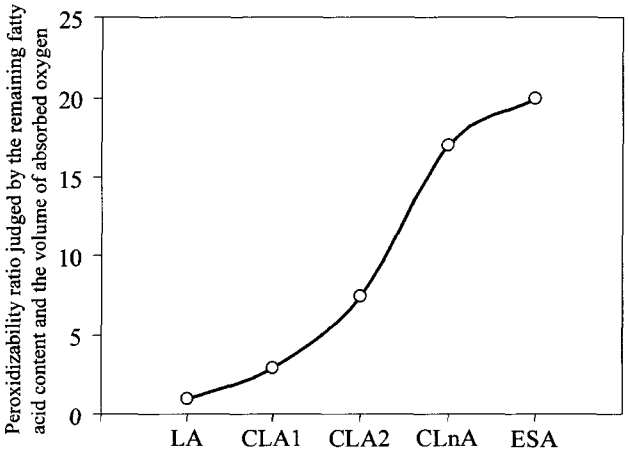

Figure 2. Ease of peroxidation of conjugated fatty acids *in vitro*. The activity was compared with LA. LA = linoleic acid (9*c*12*c*-18:2), CLA1 = conjugated linoleic acid (9*c*11*t*-18:2), CLA2 = conjugated linoleic acid (10*t*12*c*-18:2), CLnA = conjugated linolenic acid (mixtures of isomers prepared by alkaline isomerization), ESA = α-eleostearic acid (9*c*11*t*13*t*-18:3).

of the remaining fatty acid content and the volume of absorbed oxygen, these conjugated fatty acids appear to be easily oxidized. However, based on the contents of lipid hydroperoxide and thiobarbituric acid reactive substances (TBARS), they were still oxidized less than the reference unconjugated fatty acids. These results suggest that conjugated fatty acid itself causes little oxidative stress *in vitro* because the oxidative stress is caused by the lipid hydroperoxide which is the first oxidation product and TBARS which is the second oxidation product.

Next, we investigated the oxidative stability of the triacylglycerol form of conjugated fatty acid (conjugated α-linolenic acid), because this form

may be suitable for application in foods. The susceptibility to peroxidation of the triacylglycerol form was lower than that of unesterified ('free') conjugated α-linolenic acid. This suggests that oxidative stability of conjugated fatty acid is improved by protecting the carboxylic acid group through esterification.

To further improve the oxidative stability of the triacylglycerol form of conjugated α-linolenic acid, we added the well-known, fat-soluble antioxidant α-tocopherol at 0.1% of sample weight. As a result, its oxidative stability was increased to that of the triacylglycerol form of α-linolenic acid.

The results of this *in vitro* study showed that conjugated fatty acids (α-eleostearic acid, conjugated α-linolenic acid and CLA) in their free fatty acid forms were easily oxidized, with the following ranking: α-eleostearic acid > conjugated α-linolenic acid > CLA. However, lipid hydroperoxide and TBARS contents after oxidation of conjugated fatty acids were low, suggesting production of only small amounts of rapid-reacting secondary oxidation products. Therefore, if conjugated fatty acids are used in foods or medicines, the means to prevent these from the oxidation may be necessary. Using conjugated fatty acids in triacylglycerol ester form and in the presence of an antioxidant ensures their stability for the oxidation, and thus might use their bioavailability effectively.

Induction of apoptosis by conjugated fatty acids in cell culture

Conjugated fatty acids (α-eleostearic acid, conjugated α-linolenic acid and CLA) were easily oxidized *in vitro*. In cell culture, they showed intensive cytotoxicity to tumour cell lines (2). For instance, α-eleostearic acid and conjugated α-linolenic acid exhibited cytotoxicity with LD_{50} at 10 and 20 μM, respectively, in DLD-1 cells (colorectal adenocarcinoma). Interestingly, they had no harmful effect on normal human fibroblast cell lines such as MRC-5, TIG-103, and KMS-6 cells.

The cytotoxic action of α-eleostearic acid and conjugated α-linolenic acid was also demonstrated in other tumour cell lines including HepG2, A549, MCF-7, and MKN-7 cells. α-Tocopherol suppressed cytotoxicity of α-eleostearic acid and conjugated α-linolenic acid in tumour cells, and the cytotoxicity involved membrane phospholipid peroxidation. α-eleostearic acid and conjugated α-linolenic acid induced DNA condensation and fragmentation in DLD-1 cells, indicating the involvement of apoptosis in this cytotoxic mechanism.

These results show that conjugated fatty acids (α-eleostearic acid and conjugated α-linolenic acid) cause cell apoptosis through lipid peroxidation. The ability of the conjugated fatty acids to induce tumour cell apoptosis has the following ranking: α-eleostearic acid > conjugated α-linolenic acid > CLA (**Figure 3**). On the other hand, we recently found that conjugated α-linolenic acid inhibited mammalian DNA polymerase and topoisomerase I and II activities, which could also contribute to the therapeutic potential of conjugated fatty acids as anti-cancer compounds (7, 8).

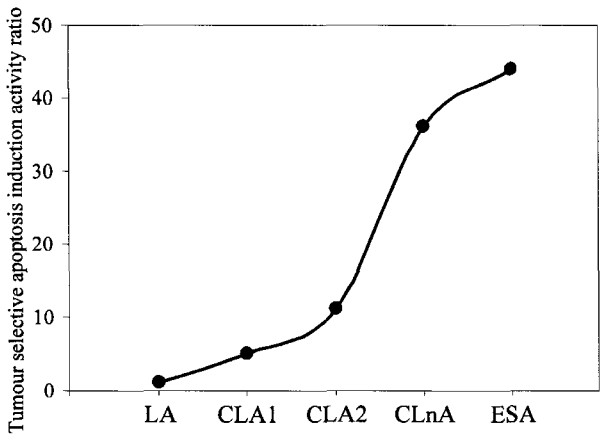

Figure 3. Tumour selective apoptosis induction activity of conjugated fatty acids *in vitro* and *in vivo*. The activity was compared with LA. LA = linoleic acid (9*c*12*c*-18:2), CLA1 = conjugated linoleic acid (9*c*11*t*-18:2), CLA2 = conjugated linoleic acid (10*t*12*c*-18:2), CLnA = conjugated linolenic acid (mixtures of isomers prepared by alkaline isomerization), ESA = α-eleostearic acid (9*c*11*t*13*t*-18:3).

In another experiment, we synthesized a newly

conjugated triene 5*t*7*t*9*t*14*c*17*c*-EPA and found it could induce apoptosis in DLD-1 cells. Such synthesized conjugated EPA may find applications in food and medical products (9).

Tumor growth inhibition by conjugated fatty acids in vivo

To verify the *in vivo* anti-tumour effect of conjugated fatty acids, we transplanted DLD-1 cells into nude mice and fed the mice with conjugated α-linolenic acid, which, together with CLA, is one of the strongest inducer of apoptosis in our tumour cell line experiment (Figure 3). Compared with CLA and α-linolenic acid (LnA), administration of conjugated α-linolenic acid showed the greatest inhibition of DLD-1 induced tumour development (2-5). Moreover, conjugated α-linolenic acid showed the highest levels of membrane phospholipids hydroperoxide and TBARS in tumours, suggesting that apoptosis was induced through lipid peroxidation as we mentioned above in connection with the cell culture study.

Conjugated α-linolenic acid had no harmful effect on normal liver tissue or plasma. It does not affect normal tissues, even at the high concentrations needed to affect tumour cells. Therefore, conjugated α-linolenic acid could be applied in food and medical products with no damage to normal tissues.

Next, we compared α-eleostearic acid and CLA in DLD-1 transplanted nude mice (2-5) and found that α-eleostearic acid had a stronger anti-tumour effect than did CLA. As shown for conjugated α-linolenic acid above, induction of apoptosis by α-eleostearic acid was consistent with enhancement of DNA fragmentation, increase in lipid peroxidation, activity of caspase that is apoptosis performance factor, and expression of caspase mRNA. Ability of the conjugated fatty acids to inhibit tumour growth was in the following ranking: α-eleostearic acid > conjugated α-linolenic acid > CLA. Furthermore, there is a recent finding indicating that conjugated EPA and conjugated DHA inhibited growth of colo 201 cancer cells transplanted in nude mice, and growth of KPL-1 tumour cells in the athymic mouse system (7, 8).

In the nude mice fed α-eleostearic acid, 9*c*11*t*-CLA was detected in plasma and tissues. We have previously shown that α-eleostearic acid is metabolized and converted to 9*c*11*t*-CLA in rats fed α-eleostearic acid. Hence, we originally concluded that α-eleostearic acid was metabolized and converted to 9*c*11*t*-CLA in mice and that CLA suppressed tumour growth (4, 5, 10). However, a much stronger suppression effect on tumour growth was observed in mice fed α-eleostearic acid relative to those fed 9*c*11*t*-CLA. The result suggest that most of the α-eleostearic acid was metabolized to 9*c*11*t*-CLA but that non-metabolized α-eleostearic acid has a strong effect on tumour cells.

Conclusions

Our *in vitro* study showed that the oxidation rate of α-eleostearic acid was faster than that of the unconjugated fatty acids and CLA. In cell culture study, by using the DLD-1 cell line, conjugated fatty acids (conjugated α-linolenic acid and α-eleostearic acid) were demonstrated to be novel protective chemotherapeutic agents, operating through lipid peroxidation to destroy tumour cells. This is probably because these conjugated fatty acids are highly susceptible to oxidation. In an *in vivo* study using DLD-1 transplanted nude mice, conjugated α-linolenic acid and α-eleostearic acid showed a stronger anti-tumour effect than CLA. Consequently, we can conclude that the oxidative stability of conjugated fatty acids is closely related to their significant anti-tumour activity.

Prospective

For the application of conjugated fatty acids (especially conjugated α-linolenic acid and α-eleostearic acid) in humans, the dosage is necessarily important. At present, CLA is the only conjugated fatty acid that has been investigated

in humans. And there is no report showing an inhibitory effect of CLA on tumour growth in humans. Administration of CLA to humans at a dose of 4 g/day for 9-12 weeks was reported to show no positive effects. It is difficult to take conjugated fatty acid by 4 g/day or more from the safety of humans. Since the human dose equivalent to the dose given to mice in our in vivo study would be more than 4 g/day, it would be necessary to study conjugated α-linolenic acid or α-eleostearic acid administration at a dose of less than 4 g/day. Moreover, it is difficult to ingest conjugated fatty acids (including CLA) from conventional, unsupplemented foods at a dose of 4 g/day and it would be preferable to make these acids available in the form of added dressings or supplements. Furthermore, it is feasible that a combination of the conjugated fatty acids with other bioactive agents may provide an even stronger anti-tumour effect, even at lower doses, and this should also be investigated.

In this paper, the correlation between anti-tumor effect and oxidative stability of the conjugated fatty acid were proven. In human body, the antioxidant like α-tocopherol (Toc) exists in a high density. Therefore, there is a possibility that the activity of anti-tumor effect changes greatly depending on the activated structure of the conjugated fatty acid. It is reported that the simultaneous uptake of an anticancer agent and high density Toc promotes the progress of cancer by the epidemiology and the clinical trial. It is clarified that these anticancer agents kill the cancer cell by the oxidative stress, and this is almost the same as the mechanism of the anti-tumor effect of the conjugated fatty acid. Therefore, the high intake of Toc might deny the anti-tumor effect of the conjugated fatty acid. Moreover, the activated structure of the conjugated fatty acid is not clarified, the further research is necessary for the decision of the activated structure.

It is very difficult to separate and purify CLA from natural sources owing to the low concentrations present. Therefore, CLA mixtures prepared by alkaline isomerization are currently available on the market as health supplements. In contrast, α-eleostearic acid can be purified with relative ease from tung oil and goya (*Momordica charantia*; a popular vegetable in the Okinawa region of Japan). Furthermore, some oilseeds contain α-eleostearic acid or conjugated α-linolenic acid as almost the single constituent fatty acid. Consequently, it is quite easy to conduct studies using this oil.

We would expect α-eleostearic acid to be superior to CLA as a dietary supplement, once its safety is confirmed. It has potential in therapeutic applications, because our research results suggest that it is more effective than CLA at suppressing tumour growth.

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共役脂肪酸：酸化安定性と生理機能の関係

都築　毅*

概　要

共役リノール酸は脂質生化学や脂質栄養学の分野においてよく知られた存在である。また、天然には共役リノール酸以外の共役脂肪酸も存在する。例えば、共役リノール酸より多価不飽和である共役リノレン酸は、キリやニガウリの種子に含まれている。私は共役脂肪酸の特異的な生理機能に興味をもち、様々な研究をしてきた。そして、共役脂肪酸の酸化安定性試験において、共役二重結合の数が増えると酸化安定性が減少することを見出した。つまり、共役リノール酸より共役リノレン酸の方が早く酸化されるということである。また、培養細胞や動物試験にて、共役脂肪酸の共役二重結合の数が増えると癌抑制効果が増大することを見出した。これらの結果から共役脂肪酸の酸化安定性と癌抑制効果に、何らかの関係があるように思われた。よって、本論文において共役脂肪酸の酸化安定性と癌抑制効果との間にどのような関係があるのかを調べた。そして、共役脂肪酸の酸化安定性と癌抑制効果の間には密接な関係があるという結論に達した。

キーワード：共役脂肪酸、共役リノール酸、共役リノレン酸、エレオステアリン酸、脂質過酸化