Dose-Dependent Effect of Dietary Conjugated Linoleic Acid on the Growth of Rat Hepatoma dRLh-84 Cells In Vivo

Masao Yamasaki, Atsushi Ikeda, Akira Hirao, Yoko Tanaka, Tatsuya Rikimaru, Mitsuo Shimada, Keizo Sugimachi, Hirofumi Tachibana and Koji Yamada*

Laboratory of Food Chemistry, Division of Applied Biological Chemistry, Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, 6–10–1 Hakozaki, Higashi-ku, Fukuoka 812–8581, Japan
1 Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, 3–1–1, Maidashi, Higashi-ku, Fukuoka 812–8582, Japan
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Summary In this study, the effect of varying doses of conjugated linoleic acid (CLA) on the growth of transplanted hepatoma dRLh-84 cells and the relationship between tumor growth and prostaglandin (PG) E2 production or cyclooxygenase (COX)-2 expression were examined. Donryu rats were fed an experimental diet containing 0, 0.1, 0.5, or 2 wt.% CLA for 3 wk, and then dRLh-84 cells were transplanted into the liver. Results show that dietary CLA (0.5 and 2 wt.%) significantly enhanced the growth of the transplanted hepatoma cells compared to the non-CLA diet group at 20 d after cell transplantation. Tumor weight at 10 d after transplantation was also significantly higher in the 2 wt.% CLA group than in non-CLA fed rats. Ten days after transplantation, the PGE2 level in the tumor tissue was shown to be depressed in a CLA dose-dependent manner. Cyclooxygenase-2 (COX-2) mRNA expression in the tumor also tended to be lower in the CLA group than in the non-CLA diet group 10 d after transplantation. Dietary CLA did not affect the tumor phospholipid arachidonic acid level, which is a substrate for PG synthesis. These results indicate that dietary CLA of at least 0.5 wt.% enhances the growth of transplanted dRLh-84 cells in vivo. It is believed that growth promotion of dRLh-84 cells in vivo by CLA cannot be clarified by the PG synthesis dependent mechanism.

Key Words conjugated linoleic acid, dRLh-84, hepatoma, prostaglandin, cyclooxygenase

Conjugated linoleic acid (CLA) is a generic term for geometric and positional isomers of octadecadienoic acid. It has been reported that CLA has various beneficial physiological effects, such as the inhibition of chemically induced carcinogenesis in various tissues such as the mammary gland (1–3), skin (4), forestomach (5), and intestine (6). Moreover, it has been reported that CLA inhibits the proliferation of various cancer cell lines in vitro (7, 8) as well as the proliferation or induction of cell death in human liver tumor cell lines such as HepG2, SNU-128, and 7800NJ cells (9–12). On the other hand, we previously reported that dietary CLA (2 wt.%) enhanced tumor growth of transplanted hepatoma dRLh-84 cells in Donryu rats (13). This data indicates that a high dose (2 wt.%) of dietary CLA promotes the growth of transplanted dRLh-84 rat hepatoma cells. Most in vivo studies of CLA have been performed beyond 1 wt.% of diet, yet a volume of only 0.1 wt.% dietary CLA was an effective dose to inhibit chemically induced carcinogenesis (1–6). However, the effect of lower doses of dietary CLA on transplanted hepatoma cells has not been clearly elucidated.

Cyclooxygenase (COX) has been shown to be a key enzyme in the production of prostaglandins (PGs) from arachidonic acid, and two isoforms of COX have been reported (14, 15). Among them, COX-1 is constitutively expressed in various tissues, while COX-2 is an inducible enzyme and can be induced by various stimulators such as lipopolysaccharide, cytokines and growth factors (16, 17). It has been reported that many cancer cell lines strongly express COX-2 (18, 19). COX-2 is also highly expressed in hepatocellular carcinoma (HCC) cells and considered to be related to tumor cell differentiation (20, 21). Moreover, it has been reported that the elevated levels of PGE2 in tumor tissue are because of high COX-2 expression (22–24). Thus, COX-2 expression and production of PGE2 are expected to be key regulators of hepatocyte and liver tumor cell growth. In this study, we examined the effect of various doses of CLA on transplanted dRLh-84 hepatoma cells and the relationship between tumor growth and PGE2 production or COX-2 expression.
MATERIALS AND METHODS

Experimental animal and diet. CLA and safflower oil (SAF) were prepared by Rinoru Oil Mills Co., Ltd. (Nagoya, Japan). Compositions of CLA were as follows: 9c,11t, 46.2%; 10c,12c, 47.5%; 9c,11c and 10c,12c, 3.1%; 9t,11t and 10t,12t, 3.3%. Four-week-old male Donryu rats (n=64) were purchased from Charles River Japan Inc. (Yokohama, Japan), fed a non-purified diet freely, and given water ad libitum for 1 wk after arrival. After the acclimatization period, rats were separated into four groups of 16 rats each, and then the experimental diet was initiated. Experimental diets were prepared according to AIN-93G and contained 7wt.% SAF+0.5 wt.% CLA (0 wt.% CLA), 6.9 wt.% SAF+0.1 wt.% CLA (0.5 wt.% CLA), or 5 wt.% SAF+2 wt.% CLA (2 wt.% CLA). Rats were kept on a 12 h light/12 h dark (8:00 to 20:00) cycle in a 20°C environment and under specific pathogen-free conditions.

After a 3 wk of feeding, dRLh-84 cells suspended in phosphate-buffered saline (PBS) were injected into the left lobe of the hepatic capsule at 1.0×10^6 cells/rat (injected group). Other rats in each dietary group were injected with PBS only (control group). Other rats in each dietary group were in a 20°C environment and under specific pathogen-free conditions.

RT-PCR and Southern blot analysis. Isolation of total RNA in the tumor portion was performed using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the appended protocol. Tissues in the same experimental groups were pooled before RNA isolation. For cDNA synthesis, isolated total RNA was denatured at 70°C for 10 min and chilled on ice for 10 min. Then, reverse-transcription was performed in a reaction solution containing 1.0 ml dNTP, 20 units Moloney mouse leukemia virus (MMLV)-reverse transcriptase (Amersham Pharmacia Biotech, Buckinghamshire, UK), and 0.1 unit RNase inhibitor at 37°C for 1 h. Synthesized cDNA was used to perform PCR amplification in the presence of COX-2 or β-actin specific primers. Sequences of the primers used are as follows: COX-2, sense 5'-GAGCATCAAGAGATGTGATCGAGA-3' and anti-sense 5'-AACGATGCGTCACCTCAATG-3'; β-actin, sense 5'-TGAACATAGGCAACCGT-3' and anti-sense 5'-GCCAGGCACGGCATG-3'. For PCR, temperatures established were: 95°C for denaturation, 50°C for annealing, and 72°C for polymerizations, and 30 cycles (both β-actin and COX-2) were performed. The amplified products were subjected to electrophoresis on a 1.5% agarose gel and then transferred to a Hybond N+ membrane. Sequences for the oligoprobes are as follows: COX-2, 5'-AAGCAGCTCTGGGTCGAAC-3' and; β-actin, 5'-TTGAGCCAGGGCCAT-3'. After a 3 wk of feeding, dRLh-84 cells suspended in phosphate-buffered saline (PBS) were injected into the left lobe of the hepatic capsule at 1.0×10^6 cells/rat (injected group). Other rats in each dietary group were injected with PBS only (control group). Other rats in each dietary group were in a 20°C environment and under specific pathogen-free conditions.

To prevent the post-excision expression of PGs, excised tumor portions were homogenized with 0.1 M Tris-HCl containing 1 mm indomethacin on ice. Then, homogenates were adjusted to pH 3.5 using 1N HCl and centrifuged at 5,000×g for 10 min. The supernatants were recovered and loaded slowly on Sep-Pak C-18 cartridges (Waters, Milford, MA, USA). Columns were washed with diluted water, 15% ethanol, and benzene in this order, and then PGs were eluted using methanol : ethyl acetate (1:9) solution. Afterwards, the eluted eicosanoid fraction was labeled using 9-anthrildiazomethane (ADAM) (Funakoshi Co., Ltd., Tokyo, Japan). Briefly, 0.2% ADAM solution was mixed with the PGs solution in pentane for 20 min at 40°C and loaded on Sep-Pak SIL (Waters). Sep-Paks were washed with a chloroform : toluene (1:1) solution and ADAM labeled PGs were eluted with an acetonitrile : methanol (4:1) solution. Eluted solutions were evaporated in a vacuum and resolved in a methanol : ethyl acetate (1:1) solution for application on a spectrofluorometric HPLC.

To analyze ADAM labeled PGE2, LC-Module I (Waters) equipped with a YMC-Pack ODS A-312 column (YMC, Tokyo, Japan) was used. A mobile phase acetonitrile : H2O : acetic acid (70:30:1) solution was adopted and adjusted to 1.0 ml/min. PGE2 was detected using the RF-10A spectrofluorometric detector (EX 365 nm, EM 412 nm) (Shimadzu, Kyoto, Japan).

RESULTS

Growth parameters

At the start of the feeding period, the average weight of rats in each group was set at 153 g and there was no
Table 1. Dietary effect of conjugated linoleic acid on the liver weight of Donryu rats.

<table>
<thead>
<tr>
<th>Dietary CLA (%)</th>
<th>0</th>
<th>0.1</th>
<th>0.5</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.08±0.04</td>
<td>3.35±0.05</td>
<td>3.46±0.03</td>
<td>3.76±0.14</td>
</tr>
<tr>
<td>Injected tumor-</td>
<td>3.20±0.08</td>
<td>3.10±0.10</td>
<td>3.16±0.05</td>
<td>3.33±0.04</td>
</tr>
<tr>
<td>Injected tumor+</td>
<td>3.37±0.09</td>
<td>3.30±0.12</td>
<td>3.36±0.04</td>
<td>3.70±0.10</td>
</tr>
<tr>
<td>Control</td>
<td>3.03±0.14</td>
<td>3.08±0.18</td>
<td>3.46±0.18</td>
<td>4.09±0.16</td>
</tr>
<tr>
<td>Injected tumor-</td>
<td>2.91±0.07</td>
<td>2.98±0.02</td>
<td>3.07±0.05</td>
<td>3.19±0.09</td>
</tr>
<tr>
<td>Injected tumor+</td>
<td>3.60±0.27</td>
<td>4.07±0.30</td>
<td>5.12±0.16</td>
<td>5.23±0.35</td>
</tr>
</tbody>
</table>

ANOVA

<table>
<thead>
<tr>
<th>Effect</th>
<th>Liver tumor-</th>
<th>Liver tumor+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLA (1)</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Injection (2)</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Duration (3)</td>
<td>p=0.155</td>
<td>p=0.979</td>
</tr>
<tr>
<td>(1)×(2)</td>
<td>p&lt;0.001</td>
<td>p=0.137</td>
</tr>
<tr>
<td>(1)×(3)</td>
<td>p=0.251</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>(2)×(3)</td>
<td>p=0.133</td>
<td>p=0.519</td>
</tr>
<tr>
<td>(1)×(2)×(3)</td>
<td>p=0.265</td>
<td></td>
</tr>
</tbody>
</table>

Data are means±SE for three or five rats in control and injected groups, respectively. Statistical analysis was performed using a 3-way ANOVA test to evaluate the significance of difference.

Table 2. Dietary effect of conjugated linoleic acid on the growth of transplanted hepatoma dRLh-84 cells.

<table>
<thead>
<tr>
<th>After injection</th>
<th>0</th>
<th>0.1</th>
<th>0.5</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 d</td>
<td>0.53±0.05</td>
<td>0.60±0.10</td>
<td>0.61±0.18</td>
<td>1.16±0.27</td>
</tr>
<tr>
<td>20 d</td>
<td>2.33±0.85</td>
<td>3.67±1.10</td>
<td>7.14±0.56</td>
<td>6.69±1.34</td>
</tr>
</tbody>
</table>

Data are means±SE for four or five rats in each group. Values without any common superscript letter are significantly different from each other at p<0.05.

significant difference in weight gain and food efficiency among all the dietary groups (data not shown). The weights of heart, kidneys, lungs, and spleen at the end of the feeding period were also comparable among all the dietary groups (data not shown).

The liver weight is presented as weight with or without tumor (expressed as tumor- or tumor+) in Table 1. At 10 and 20 d post-injection, a single solid tumor was formed in all injected rats. The tumor-liver weight increased in a dose dependent manner for the CLA group, irrespective of cell injection. Tumor- liver weights in the control rats were significantly higher than the injected rats, p<0.001. In the injected rats, the tumor+ liver weight tended to be high in the CLA groups, especially for 0.5 and 2 wt.% on day 20 after injection, because tumor weights were generally higher in CLA groups than in the 0 wt.% CLA group (details shown in Table 2).

Tumor weight

Table 2 shows the effect of varying doses of CLA on tumor weight after the transplantation of dRLh-84 cells. In control rats, no liver tumor was found. In injected rats, the cells formed a single solid tumor on the liver at least 10 d after the injection. At that time, tumor weight was slight and not significantly higher in the 0.1 and 0.5 wt.% CLA groups than in the 0 wt.% CLA group. On the other hand, tumor weight in the 2 wt.% CLA rats was more than twofold compared to the 0 wt.% CLA rats. On day 20 after the injection, tumor weight increased in a dose-dependent manner in the CLA-fed rats and reached a plateau at the 0.5 wt.% dose. There was no significant difference between the 0.5 and 2 wt.% CLA groups.

PGE2 level in the tumor tissue

The effect of CLA on the tumor PGE2 level is shown in Table 3. On day 10 after injection, the PGE2 level was shown to have decreased in a dose-dependent manner in CLA-fed rats. The PGE2 level in the 2 wt.% CLA group was less than 12% of the 0 wt.% CLA group and this difference was statistically significant. On day 20
Table 3. Dietary effect of conjugated linoleic acid on the prostaglandin E2 level in the tumor tissue.

<table>
<thead>
<tr>
<th>After injection</th>
<th>PGE2 (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>10 d</td>
<td>499±145a</td>
</tr>
<tr>
<td>20 d</td>
<td>216±60</td>
</tr>
</tbody>
</table>

Data are means±SE for four or five rats in each group. Values without any common superscript letter are significantly different from each other at p<0.05.

Fig. 1. Dietary effect of conjugated linoleic acid on the mRNA expression of cyclooxygenase-2 in the tumor tissue. A: Expressions of COX-2 and β-actin was assessed by RT-PCR and Southern blot analysis. B: Intensity of COX-2 expression was standardized as a relative value to the intensity of β-actin expression. Tissues in the same experimental groups were pooled before RNA isolation.

after injection, PGE2 levels decreased in all the dietary groups compared to the values obtained at 10 d post-injection. Moreover, there was no significant difference among all the dietary groups at day 20 after injection. COX-2 mRNA expression in the tumor tissue

Expression of COX-2 in the tumor tissue was presented as relative intensity to β-actin mRNA expression and is shown in Fig. 1. At 10 d post-injection, COX-2 mRNA expression was less in the 0.5 and 2 wt.% CLA groups than in the control group. The unit value for the 0.1 wt.% CLA group was much the same as that for the 0 wt.% CLA. On the other hand, the seemingly CLA dependent COX-2 mRNA expression inhibition was not observed on day 20 after injection. Namely, no regular relationship between COX-2 expression and CLA dosage could be demonstrated.

Fatty acid composition in tumor phospholipids

Table 4 shows the effect of CLA on phospholipid fatty acid composition and CLA accumulation in the tumor. As for the arachidonic acid level, there was no significant difference among all dietary groups irrespective of feeding duration. No marked fluctuation was found in any other major saturated, monounsaturated or polyunsaturated fatty acid composition in the phospholipids among all the dietary groups. CLA accumulation was recognized only in the 0.5 wt.% CLA group on day 20 after injection, and no CLA peak was detected by gas chromatogram in any of the other groups.

DISCUSSION

It has been reported that CLA inhibits chemically induced carcinogenesis in various tissues (1–6). In vitro studies show that CLA also inhibits the proliferation of various tumor cell lines derived from various tissues (7, 8) and several human liver cancer cell lines (9–12). Conversely, CLA has been reported to cause liver enlargement and enhance hepatic ornitine decarboxylase activity in mice (28); consequently, CLA may act as hepatocyte proliferator. However, the effects of dietary CLA on hepatic carcinogenesis and liver tumor growth have not been clearly elucidated. Previously, we revealed that dietary CLA (2 wt.%) promoted transplanted hepatoma dRLh-84 cell growth in vivo (13). In this report, we demonstrated that not only 2 wt.%, but also 0.5 wt.% dietary CLA significantly promoted transplanted dRLh-84 cell growth in vivo.
It has been reported that 9c,11t-CLA or linoleic acid promote proliferation and 10t,12c-CLA induces potent cytotoxicity in dRLh-84 cells in vitro (29). In addition, it has been revealed that 9c,11t-CLA was preferentially accumulated in tumor tissues compared to 10t,12c-CLA (Yamasaki et al., unpublished data). Oppositely, we observed hardly any CLA accumulation in tumor phospholipids at any dose or feeding duration, so it is believed that dietary CLA is harder to accumulate into tissue phospholipids. These data suggest 9c,11t-CLA may be preferentially accumulate into tumor tissues, resulting in the growth promotion of transplanted dRLh-84 cells. Further studies are needed to clarify the mechanism of growth promotion by 9c,11t-CLA in dRLh-84 cells.

As shown in Table 3, dietary CLA reduced the PGE2 level in tumor tissue, and it was assumed that the down-regulation of COX-2 mRNA expression partly contributed to the reduction of PGE2. The high expression of COX-2 is often recognized in cancerous tissues or cancer cells, and excess PG synthesis is closely related to tumor growth in various tissues, especially in the colon (30). In addition, COX-selective inhibitor has been reported to bring growth arrest in hepatocellular carcinoma (HCC) (31, 32). Moreover, COX-2 expression is highly recognized in HCC and its intensity is associated with a differentiate phenotype (20, 21, 31, 32). In this study, dietary CLA inhibited COX-2 mRNA expression and PGE2 level in transplanted cancerous tissue, but did not inhibit its growth. Taken together, PGE2 reduction and down-regulation of COX-2 mRNA expression in tumor tissue by CLA did not bring about tumor-reducing activity in this study.

Here, not only transplanted dRLh-84 cells, but also other cells such as normal hepatocytes, Kupffer cells, and endothelial cells contributed to the accumulation of PG in the tumor. Harris et al. reported that dietary CLA depressed the PGE2 and PGF2α levels in rat liver under a high n-6 polyunsaturated fatty acid diet (34). Most of the PGs produced in the liver under normal conditions do not derive from hepatocytes, but rather from nonparenchymal cells (35), and COX-1 is a dominant PG synthesis enzyme because of the low expression of COX-2 (36). Therefore, CLA might inhibit not only COX-2, but also COX-1 derived PG synthesis.

As shown in Table 4, however, the accumulation of CLA in tumor phospholipids was very low or not significantly different irrespective of CLA feeding and dosage up to 2 wt.%. Hence, it is thought that the accumulation of CLA is not important to inhibit PG synthesis. The 10t,12c CLA was also one of the major isomers in this experiment. This isomer is metabolized to 5c,8c,11t,14c eicosatetraenoic acid, and this metabolite has been reported to act as a powerful competitive inhibitor for the metabolism of arachidonic acid to PG (37). This CLA metabolite could be a putative candidate for being a PG synthesis inhibitor. In addition, PGs are synthesized from free arachidonic acid derived from membrane phospholipids. Thus, the phospholipid arachidonic acid level is expected to be a key factor in regulating tissue PG level. It has been reported that CLA converted to desaturated and elongated conjugated fatty acid, and is expected to interfere in the n-6 polyun-
saturated fatty acid metabolism (38). On the other hand, our data shows that CLA does not affect the phospholipid arachidonic acid level in tumor tissue. In addition, the docosapentaenoic acid (DPA) level was significantly lower in all CLA groups as compared to the control group at 10 and 20-d post-injection (Table 4). It is well known that DPA is converted to arachidonic acid through the β-oxidation pathway, and its level is known to decrease malignant prostate tissue but to increase in mononuclear blood cells in leukemia patients (39, 40). Thus, arachidonic acid metabolites containing DPA may play an important role in tumor cell proliferation. As a result, dietary CLA is believed to reduce the PGE2 level in tumor tissue; however, not by reduction of arachidonic acid level in the tumor portion.

We previously reported that tumor growth promotion by dietary CLA (2 wt.%) coincided with the elevation of serum tumor necrosis factor (TNF-α) and interferon (IFN)-γ (13). These results suggest that dietary CLA caused an inflammatory response in tumor-bearing rats. COX-2 expression is well known to be induced by some cytokines like TNF-α and IFN-γ (41, 42). Dietary CLA inhibited PG synthesis as described above on day 10 after injection, but it was considered that the production of inflammatory cytokines induced by dietary CLA might cancel the inhibitory effect of CLA on COX-2 mRNA expression at day 20 after injection.

In conclusion, it has been revealed that 0.5 wt.% dietary CLA enhances the growth of transplanted dRLh-84 cells and slight growth stimulation was recognized when using 0.1 wt.% CLA. However, we could not observe a positive relationship between tumor growth and PGE2 level or the intensity of COX-2 mRNA expression. Hence, the growth of transplanted dRLh-84 cells may not be strongly dominated by certain PGE2 levels or COX-2 expression. To examine the effects of CLA on the growth of other hepatoma and hepatic carcinogenesis, more studies are needed.

REFERENCES


Effect of CLA on the In Vivo Growth of Hepatoma


