Decrease in linoleic acid metabolites as a potential mechanism in cancer risk reduction by conjugated linoleic acid

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Conjugated linoleic acid (CLA) is a term used to denote certain positional isomers of linoleic acid (3). Linoleic acid is an 18 carbon unsaturated fatty acid with two double bonds at positions 9 and 12. In contrast, the two double bonds in CLA are at positions 9 and 11 or 10 and 12, thus giving rise to the designation as a conjugated diene. Milk and other dairy products are good sources of CLA (4) because of the unique metabolic capability of rumen bacteria in converting linoleic acid to CLA via an enzymatic isomerisation reaction (5,6). Almost all of the biological research with CLA was done using a commercial preparation which contains a mixture of the 9,11 and 10,12 isomers, although CLA in food is present predominantly as the 9,11 isomer. Despite the similarity in structure between linoleic acid and CLA, their impact on mammary cancer development is strikingly different. In contrast to linoleic acid, which is known to stimulate carcinogen-induced mammary carcinogenesis, CLA is effective in decreasing mammary tumor yield produced by 0.5 and 1% CLA. No further decrease in either parameter was observed when CLA in the diet was raised to 1.5 or 2%. Thus, optimal CLA nutrition during pubescence could conceivably control the population of cancer-sensitive target sites in the mammary gland. Since both CLA and linoleic acid are likely to share the same enzyme system for chain desaturation and elongation, it is possible that increased CLA intake may interfere with the further metabolism of linoleic acid. Fatty acid analysis of total lipid showed that CLA and CLA metabolites continued to accumulate in mammary tissue in a dose-dependent manner over the range 0.5–2% CLA. There was no perturbation in tissue linoleic acid, however, linoleic acid metabolites (including 18:3, 20:3 and 20:4) were consistently depressed by up to 1% CLA. Of particular interest was the significant drop in 20:4 (arachidonic acid), which is the substrate for the cyclooxygenase and lipoxygenase pathways of eicosanoid biosynthesis. Thus the CLA dose–response effect on arachidonic acid suppression corresponded closely with the CLA dose–response effect on cancer protection in the mammary gland. This information is critical in providing new insights regarding the biochemical action of CLA.

Introduction

A recent epidemiological study in Finland showed that habitual consumption of whole milk is associated with a reduced risk of breast cancer (1). This was a 25 year prospective study involving 4697 women with a mean age of 39 at the time of recruitment and who were cancer free initially. Among individuals with the highest tertile of milk intake, there was a 60% decrease in relative risk. The adjustment for potential confounding factors, such as smoking, body mass index, number of childbirths, nutrients, etc., did not alter the results. It is possible that some covariant with milk was not assessed in this study and, furthermore, the design would not permit the identification of the active constituent(s) involved. Nonetheless, a growing body of evidence during the past decade suggests that milk fat may contain a number of components with anticancer activity, including conjugated linoleic acid, sphingomyelin and butyric acid (reviewed in ref. 2).

Conjugated linoleic acid (CLA) is a term used to denote certain positional isomers of linoleic acid (3). Linoleic acid is an 18 carbon unsaturated fatty acid with two double bonds at positions 9 and 12. In contrast, the two double bonds in CLA are at positions 9 and 11 or 10 and 12, thus giving rise to the designation as a conjugated diene. Milk and other dairy products are good sources of CLA (4) because of the unique metabolic capability of rumen bacteria in converting linoleic acid to CLA via an enzymatic isomerisation reaction (5,6). Almost all of the biological research with CLA was done using a commercial preparation which contains a mixture of the 9,11 and 10,12 isomers, although CLA in food is present predominantly as the 9,11 isomer. Despite the similarity in structure between linoleic acid and CLA, their impact on mammary cancer development is strikingly different. In contrast to linoleic acid, which is known to stimulate carcinogenesis over a wide concentration range (7,8), feeding of CLA at ≥1% in the diet produces a significant protective effect (9). In the last few years, several groups of investigators have reported successful cancer prevention by CLA in a number of animal models, including tumors of the mammary gland (10), forestomach (11), colon (12) and skin (13).

Previous research from the Ip laboratory showed a unique activity of CLA in mammary cancer prevention in the rat. When CLA feeding was limited to the period of pubescent mammary gland development it was able to confer a lasting protection against subsequent induction of mammary tumors (14). An evaluation of a digitized image of the mammary tree in whole mounts showed that there was diminished morphogenesis and epithelial branching as a result of CLA treatment (15). This could in part account for the reduced susceptibility to cancer induction because of a decrease in the target cell population. The pathobiology of chemical carcinogenesis in the rat mammary gland has been well delineated (16). In this model, terminal end buds (TEB) are the primary sites for the induction of mammary carcinomas. Currently there is no quantitative data on the modulation of TEB density by CLA feeding during mammary gland maturation and, more importantly, how variations in this subset of cellular structures might affect mammary cancer risk. One of...
the objectives of the present study was to investigate the dose-dependent effect of CLA on such a relationship.

In an earlier publication, Banni et al. (17) reported that CLA can be desaturated and elongated in vivo while still maintaining the conjugated diene structure. Since both CLA and linoleic acid are likely to share the same enzyme system for chain desaturation and elongation, it is possible that increased CLA intake may interfere with the further metabolism of linoleic acid. A second objective, therefore, was to determine whether the dose–response effect of CLA on a reduction in mammary cancer risk might be correlated with a particular pattern of tissue CLA and/or linoleic acid metabolites. The above knowledge is critical in providing biochemical clues regarding the mechanism of action of CLA.

Materials and methods

Animals and CLA supplementation

Pathogen-free female Sprague–Dawley rats were purchased from Charles River Breeding Laboratories at weaning. Upon arrival, they were fed either the basal AIN-76A diet (9) or the basal diet containing 0.5, 1, 1.5 or 2% CLA (Nu-Chek, Elysian, MN). All animals were kept on these five different diets for 1 month (i.e. from 23 to 55 days of age) to prepare them for three separate experiments: (i) quantitation of TEB density in the mammary gland (n = 6 rats/group); (ii) mammary carcinogenesis bioassay in which rats were injected (i.p.) with 50 mg/kg body wt methyl nitrosourea (MNU) (n = 30 rats/group); (iii) analysis of CLA, CLA metabolites, and linoleic acid metabolites in tissues (n = 6 rats/group). In experiments (i) and (ii), rats were killed after 1 month of CLA feeding; mammary gland, liver and blood were then collected from each animal at the time of necropsy. In experiment (iii), all rats were returned to the basal diet without CLA after MNU administration. They were palpated weekly for the detection of mammary tumors (9). The experiment continued for 25 more weeks before termination. By this time, the development of palpable tumors had plateaued for several weeks across all groups.

Preparation of mammary gland whole mounts

The abdominal–inguinal mammary gland chain was excised in one piece and stretched onto a 75 × 50 mm microscope slide. The whole mount was fixed in buffered formalin, dehydrated using a series of ethanol solutions and cleared with two changes of toluene. After rehydration, the tissue was stained with alum carmine. A detailed procedure for the methodology was reported previously (15). The outer 5 mm margin of the mammary whole mount was examined by light microscopy using the criteria described by Russo and Russo (18). This area represents the location of the actively proliferating TEB structures of the mammary gland for a young virgin rat.

Quantification of conjugated and non-conjugated diene polyunsaturated fatty acids

Mammary fat pad, liver and plasma were stored at −80°C until they were ready for analysis. Total lipid was extracted by the method of Folch et al. (19). Free fatty acids were obtained by a mild saponification procedure described by Banni et al. (20) and collected in n-hexane. After solvent evaporation, the residue was redissolved in CH3CN/0.14% CH3COOH (v/v) for injection into the HPLC system. Separation of unsaturated fatty acids was carried out with a Hewlett-Packard 1050 liquid chromatograph equipped with a diode array detector 1040M (Hewlett Packard, Palo Alto, CA). A C-18 Alltech Adsorbosphere column (5 µm particle size, 250 × 4.6 mm) was used with a mobile phase of CH3CN/H2O/CH3COOH (70:30:0.12 v/v/v) at a flow rate of 1.5 ml/min. Non-conjugated diene unsaturated fatty acids were detected at 200 nm and conjugated diene unsaturated fatty acids at 234 nm. Spectra (195–350 nm) of the eluates were obtained at every 1.3 s and were electronically stored. Second derivative UV spectra of the conjugated diene fatty acids were generated using the Phoenix 3D HP Chemstation software. These spectra were taken to confirm the identification of the HPLC peaks. Details of the methodology regarding the characterization of conjugated diene unsaturated fatty acids in both reference and biological samples have been published by Banni et al. (21). The method of using an HPLC diode array detector system to analyze polyunsaturated fatty acids (especially those present at low levels) is much more sensitive than the conventional GC method which gives a profile of both saturated and unsaturated fatty acids.

Statistical analyses

The CLA dose–response effect on tumor incidence and tumor yield was analyzed by logistic regression and polynomial regression, respectively, as described in a previous report (22). INSTAT software (GraphPad Software, San Diego, CA) was used to calculate the mean and standard error (SE) of fatty acid measurements. One way ANOVA was applied to evaluate the differences between CLA group means and that of the control. This program is based on the Bonferroni method which requires a higher threshold for statistical significance. The increased stringency is achieved by dividing the traditional random chance probability of 5% by the number of comparisons in the data set.

Results

Reduction in TEB density and mammary cancer risk as a function of CLA intake

Our previous study demonstrated that mammary epithelial branching, as determined by digitized image analysis, was reduced by CLA feeding (15). Since TEBs are the primary sites for the induction of adenocarcinomas in the rodent mammary gland, the first experiment was designed to evaluate the density of TEBs in rats which had been given increasing levels of CLA during the period of pubescent mammary gland development (i.e. from weaning to 55 days of age). Figure 1 shows several TEBs in a mammary whole mount (Figure 1A) and a representative histological section of this structure under high power magnification (Figure 1B). In control rats given a basal diet without CLA, there were 6 TEBs/mm2 found in the abdominal–inguinal gland, a number that is consistent with that reported by Russo and Russo (18). As can be seen in Table I, increasing the level of dietary CLA to 0.5 and 1% resulted in a graded decrease in TEB density to 5.2 and 4.5 TEBs/mm2, respectively (P < 0.05). No further decrease was observed when dietary CLA was raised to 1.5 or 2%.

What is the implication of this reduction in TEB density in relation to mammary cancer risk? Table I also summarizes the mammary carcinogenesis data in rats which were fed increasing levels of CLA for 1 month from weaning (i.e. the same protocol as in the TEB density study) and then given a single dose of MNU. No CLA was supplied to any of these animals after MNU administration. They were all returned to the control basal diet for the following 23 weeks until the experiment was terminated. There was a progressive inhibition of both tumor incidence and tumor yield as the pre-MNU feeding of CLA increased from 0.5 to 1% (P < 0.05; see Table I footnotes d and e). However, no further benefit in cancer prevention was detected above 1% CLA. As shown in Table I, when the amount of CLA was increased from 1 to 1.5 and then to 2% suppression of tumor incidence and total tumor yield appeared
to level off. Thus the anti-carcinogenic response of the mammary gland paralleled closely the reduction in TEB density of the mammary epithelium as a function of CLA intake.

Accumulation of tissue CLA and CLA metabolites as a function of CLA intake

The analytical method for quantifying CLA and CLA metabolites does not distinguish the position of the conjugated dienes (i.e. 9,11 versus 10,12 isomer). Since the commercial CLA preparation used in this study consists of an equal mixture of the 9,11 and 10,12 isomers (9), the analytical data could not provide information on the possible selective incorporation of one CLA isomer over the other or any differences in the conversion of individual isomers to the longer chain metabolites. Thus the results should be interpreted with this limitation in mind even though the terms ‘CLA’ and ‘CLA metabolites’ are used here in an inclusive sense to denote specific fatty acids with the conjugated diene structure. In other words, the CLA metabolites reported in the table could have originated from the 9,11-CLA and/or 10,12-CLA, the proportion of which are used here in an inclusive sense to denote specific fatty acids with the conjugated diene structure. In other words, the CLA metabolites reported in the table could have originated from the 9,11-CLA and/or 10,12-CLA, the proportion of which remains unknown with the present method of detection.

Figure 2 shows the progressive elevation of CLA in mammary fat pad, liver and plasma as dietary CLA increased over the range 0.5–2% (P < 0.05 in all three tissues). In control rats not receiving CLA, tissue CLA concentrations were either undetectable or barely above the detection limit. For this reason, the control values are not presented in the figure. Compared with the liver, the mammary gland had a higher CLA concentration on a per milligram lipid basis. This is to be expected because we have previously found significant incorporation of CLA into neutral lipids (23), which are the predominant component in mammary tissue. Considering that the mammary gland is essentially a fatty tissue consisting largely of adipocytes, the total amount of CLA stored in the mammary fat pad is substantial. We also examined peritoneal fat in these animals and found that CLA was retained at very similar concentration to that seen in mammary tissue (results not shown). The congruency of the data suggests that adipocyte neutral lipids are a major source of CLA in the body. Finally, the plasma data suggest that CLA levels in the circulation might be a good index of CLA intake.

Figure 3 shows the graded increase in CLA metabolites in mammary tissue and liver as a function of CLA intake. The two metabolites were identified as CD 18:3 and CD 20:3 (the CD prefix indicates the presence of a conjugated diene structure in the fatty acid). They represent the desaturation product (i.e. CD 18:3) and the elongation product (i.e. CD 20:3) of CLA. It is worth pointing out that CLA metabolites were present at a much lower level compared with CLA in both mammary tissue and liver, suggesting that only a small fraction of CLA was metabolized via the desaturation and elongation pathway.

We also analyzed the levels of CLA and CLA metabolites in the abdominal fat pad. The absolute concentrations of CLA, CD 18:3 and CD 20:3 found in this fat depot as a function of increasing CLA intake were very similar to those observed in the mammary tissue (see Figures 2 and 3). This is to be expected because both the mammary gland and abdominal fat pads predominantly consist of neutral lipid-containing adipocytes. Since the abdominal fat pad is not an organ of interest in our research, we choose only to mention its resemblance to the mammary tissue without showing the data.

Changes in linoleic acid metabolites as a function of CLA intake

Figure 4 shows that regardless of the level of intake, CLA did not interfere with the retention of linoleic acid in either mammary tissue or liver, suggesting that CLA was not displacing linoleic acid to any meaningful extent. However, the data imply that the body may handle linoleic acid and CLA differently. The basal diet in this study contained 5% corn oil (AIN-76A formulation). Since ~60% of the fatty acids in corn oil is linoleic acid, the basal diet therefore contained ~3% linoleic acid. As shown in Figure 4, the average linoleic acid concentrations were ~210 and 110 µg/mg lipid in mammary tissue and liver, respectively. In contrast, the data in Figure 2 show that at a level of 2% CLA in the diet, the average CLA concentrations were ~65 and 30 µg/mg lipid in mammary tissue and liver, respectively. Thus a ratio of 1.5 of linoleic acid to CLA in the diet produced a ratio of 3.2 in mammary tissue and a ratio of 3.6 in the liver. The discrepancy between the diet ratio and tissue ratio would suggest that either CLA is not taken up as efficiently as linoleic acid or that CLA is utilized at a faster rate than linoleic acid.

The desaturase and elongase enzyme systems are responsible for the sequential conversion 18:2 (linoleic acid)→18:3→20:3→20:4. Figure 5 shows the changes in

### Table I. Reduction in TEB density and mammary cancer risk by CLA as a function of intake

<table>
<thead>
<tr>
<th>Diet</th>
<th>TEB density&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tumor incidence</th>
<th>Total no. of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.0 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27/30 (90%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>85&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.5% CLA</td>
<td>5.2 ± 0.2</td>
<td>22/30 (73%)</td>
<td>67</td>
</tr>
<tr>
<td>1.0% CLA</td>
<td>4.5 ± 0.2</td>
<td>17/30 (57%)</td>
<td>44</td>
</tr>
<tr>
<td>1.5% CLA</td>
<td>4.3 ± 0.1</td>
<td>16/30 (53%)</td>
<td>38</td>
</tr>
<tr>
<td>2.0% CLA</td>
<td>4.2 ± 0.2</td>
<td>15/30 (50%)</td>
<td>37</td>
</tr>
</tbody>
</table>

<sup>a</sup>CLA feeding was started from weaning and continued for 1 month (i.e. 23–55 days of age). Mammary whole mounts were prepared from some animals (n = 6/group) for the TEB study. For the mammary carcinogenesis experiment, MNU was injected into each rat at this point (n = 30/group).

<sup>b</sup>All animals were returned to the basal diet without CLA after MNU administration and were killed 23 weeks later.

<sup>c</sup>No. of structures/mm<sup>2</sup>.

<sup>d</sup>Mean ± SE. Values from 0, 0.5 and 1% CLA groups are different from each other, P < 0.05.

<sup>e</sup>Dose-dependent decrease in tumor incidence from 0 to 1% CLA by logistic regression analysis, P < 0.05.

<sup>f</sup>Dose-dependent decrease in tumor yield from 0 to 1% CLA by polynomial regression analysis, P < 0.05.

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CLA and mammary cancer prevention

Changes in linoleic acid metabolites as a function of CLA intake

Figure 4 shows that regardless of the level of intake, CLA did not interfere with the retention of linoleic acid in either mammary tissue or liver, suggesting that CLA was not displacing linoleic acid to any meaningful extent. However, the data imply that the body may handle linoleic acid and CLA differently. The basal diet in this study contained 5% corn oil (AIN-76A formulation). Since ~60% of the fatty acids in corn oil is linoleic acid, the basal diet therefore contained ~3% linoleic acid. As shown in Figure 4, the average linoleic acid concentrations were ~210 and 110 µg/mg lipid in mammary tissue and liver, respectively. In contrast, the data in Figure 2 show that at a level of 2% CLA in the diet, the average CLA concentrations were ~65 and 30 µg/mg lipid in mammary tissue and liver, respectively. Thus a ratio of 1.5 of linoleic acid to CLA in the diet produced a ratio of 3.2 in mammary tissue and a ratio of 3.6 in the liver. The discrepancy between the diet ratio and tissue ratio would suggest that either CLA is not taken up as efficiently as linoleic acid or that CLA is utilized at a faster rate than linoleic acid.

The desaturase and elongase enzyme systems are responsible for the sequential conversion 18:2 (linoleic acid)→18:3→20:3→20:4. Figure 5 shows the changes in
linoleic acid metabolites in the mammary tissue as a function of CLA intake. In general, the trend indicates a significant drop ($P < 0.05$) in 18:3 and 20:3 up to 0.5% CLA. Of particular interest was the continuing decrease in 20:4 (arachidonic acid) up to 1% CLA. Above 1% CLA there was no further significant reduction in all three metabolites. As expected, the abdominal fat pad behaved very similarly to mammary tissue with respect to the alterations in 18:3, 20:3 and 20:4. However, these changes in linoleic acid metabolites were not seen in the liver (data not shown), suggesting that the effect of CLA on linoleic acid metabolism might be tissue specific, i.e. the effect was greater in neutral lipid-containing tissue (e.g. mammary gland and abdominal fat pad) than in phospholipid-containing tissue (e.g. liver).

**Discussion**

The results in Table I provide new insights regarding the role of CLA in pubescent mammary gland development and the accompanying modulation of mammary cancer risk. Among the many fatty acids known to influence mammary carcinogenesis (24,25), only CLA has this unique effect in down-regulating mammary epithelial growth during maturation and therefore the size of the target cell population susceptible to carcinogenesis. Computer analysis of a digitized image of a whole mount revealed a 20% reduction in overall branching of the mammary epithelium in rats fed 1% CLA (15). In this study, we were able to obtain quantitative confirmation of a 25% decrease in the density of TEBs. In the developing mammary gland, TEBs differentiate to mature alveolar buds, which in turn give rise to the mammary tree. A lower TEB density is thus consistent with reduced branching of the mammary epithelium. In contrast to alveolar buds, which may become hyperplastic upon transformation by a carcinogen, TEBs are the primary sites for induction of adenocarcinomas. Our data showing a dose-dependent parallel decrease in TEBs and mammary cancer risk produced by CLA support the conclusion that optimal CLA nutrition during pubescence may reduce the number of cancer-sensitive target sites in the mammary gland.

An important issue which remains to be addressed is whether CLA simply slows down the pace of mammary gland development or whether CLA nutrition during pubescence is able to imprint a durable suppressive effect on maturation and proliferative potential of the mammary epithelium. In other words, will the mammary gland in CLA-fed rats eventually catch up and differentiate fully to a stage comparable with that seen in control rats? If so, how will this outcome affect mammary cancer risk? These and other related questions need to be answered because they have far-reaching implications in
terms of pre-teen/teenage CLA nutrition and breast cancer prevention in adulthood.

We have learned in this study that plasma CLA is likely to be a reliable marker of CLA intake. However, the plasma data were obtained at only one time point after 1 month of CLA feeding. Both shorter and longer durations of feeding should be examined in order to evaluate the consistency of the results. We have also found that CLA and CLA metabolites (i.e. CD 18:3 and CD 20:3) accumulate progressively in mammary tissue in proportion to dietary CLA over the entire range 0.5–2%. In contrast, cancer protection by CLA maximized at 0.5–2%. In contrast, cancer protection by CLA maximized at 0.5–2%. In both cases, dietary CLA >1% produced little or no further change. It would be reasonable to expect that the biosynthesis of eicosanoids will be affected by the reduced availability of arachidonic acid. In addition to cancer prevention activity, CLA is known to modulate immune functions (33–36), atherogenesis (36) and phorbol ester-mediated events in keratinocytes (37). Eicosanoids are believed to be intimately involved in this spectrum of biological responses. Two recent studies also showed that CLA is capable of reducing the synthesis of prostaglandin E2 in cell culture (38,39). The data presented here also hint at the possibility that reduced traffic through the eicosanoid pathway may in part be involved in mediating the biological effects of CLA. More in-depth studies are needed not only to assess the modulation of eicosanoids by CLA in vivo but also to examine the specificity of different CLA isomers in interfering with this cascade of biochemical reactions.

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