

Retention of conjugated linoleic acid in the mammary gland is associated with tumor inhibition during the post-initiation phase of carcinogenesis

Clement Ip¹, Cheng Jiang², Henry J. Thompson² and Joseph A. Scimeca³

Department of Surgical Oncology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, ²Division of Laboratory Research, AMC Cancer Research Center, Denver, CO 80214 and ³Nutrition Department, Kraft Foods, Inc., Glenview, IL 60025, USA

¹To whom correspondence should be addressed

Conjugated linoleic acid (CLA) has been reported to have significant activity in inhibiting mammary carcinogenesis. A major objective of this study was to evaluate how changes in the concentration of CLA in mammary tissue as a function of CLA exposure/withdrawal were correlated with the rate of occurrence of mammary carcinomas. Rats treated with a single dose of dimethylbenz[*a*]anthracene (DMBA) at 50 days of age were given 1% CLA in the diet for either 4 weeks, 8 weeks or continuously following carcinogen administration. No cancer protection was evident in the 4 or 8 week-CLA treatment groups. Significant tumor inhibition was observed only in rats that were given CLA for the entire duration of the experiment (20 weeks). Analysis of CLA in the mammary gland showed that the incorporation of CLA was much higher in neutral lipids than in phospholipids. When CLA was removed from the diet, neutral lipid- and phospholipid-CLA returned to basal values in about 4 and 8 weeks, respectively. The rate of disappearance of neutral lipid-CLA (rather than phospholipid-CLA) subsequent to CLA withdrawal paralleled more closely the rate of occurrence of new tumors in the target tissue. It appears that neutral lipid-CLA may be a more sensitive marker of tumor protection than phospholipid-CLA. However, the physiological relevance of CLA accumulation in mammary lipids is unclear and remains to be determined. A secondary goal of this study was to investigate whether CLA might selectively inhibit clonal expansion of DMBA-initiated mammary epithelial cells with wild-type versus codon 61 mutated *Ha-ras* genes. Approximately 16% of carcinomas in the control group (without CLA) were found to express codon 61 *ras* mutation. Although continuous treatment with CLA reduced the total number of carcinomas by 70%, it did not alter the proportion of *ras* mutant versus wild-type carcinomas, suggesting that CLA inhibits mammary carcinogenesis irrespective of the presence or absence of the *ras* mutation.

Introduction

Conjugated linoleic acid (CLA*) is a minor fatty acid found preferentially in red meat and dairy products (1). The biosynthesis of CLA in ruminants is accounted for by a rumen bacterium, which is known to convert linoleic acid to stearic

*Abbreviations: CLA, conjugated linoleic acid; DMBA, dimethylbenz[*a*]anthracene; MNU, methylnitrosourea; PCR/RFLP, polymerase chain reaction-generated restriction fragment length polymorphism.

acid via CLA (2). Over the past decade, research from several laboratories has shown that CLA expresses powerful activity in cancer protection in a number of animal models (3–7). Feeding diets containing $\leq 1\%$ CLA results in a dose-dependent suppression of tumor development in the mammary gland (8). CLA appears to have a dual effect in the modulation of mammary carcinogenesis in rats. First, exposure to CLA during the window of active mammary gland morphogenesis may reduce the proliferation of epithelial end bud cells, thus conceivably rendering the target cell population less susceptible to carcinogen-induced neoplastic transformation (8,9). Second, CLA is also capable of inhibiting tumor promotion/progression (9); however, a continuous supply of CLA is required for this mechanism of action.

The above study regarding the effectiveness of CLA in blocking tumor progression was carried out in the methylnitrosourea (MNU)-induced mammary carcinogenesis model in rats fed a 5% corn oil diet (9). One objective of the experiments reported in this study was to confirm the necessity of maintaining CLA intake after cancer induction by using dimethylbenz[*a*]anthracene (DMBA)-treated rats fed a 20% corn oil diet. It was considered important to assess whether the requirement for continuous CLA feeding was dependent on the nature of the carcinogen and the fat content of the diet. Rats were therefore given CLA for a duration of either 4, 8 or 20 weeks, starting immediately after a single dose of DMBA, to evaluate the anti-carcinogenic efficacy of these various intervention regimens. The kinetics of mammary tissue CLA retention as a function of CLA exposure/withdrawal was also analyzed in order to determine the correlation between time-dependent changes in tissue concentrations of CLA and effectiveness of cancer protection.

Additionally, we were interested in finding out whether CLA might selectively inhibit the clonal expansion of DMBA-initiated cells carrying either the wild type or codon 61 mutated *Ha-ras* gene. Previous work from Thompson's laboratory has shown that high dietary levels of linoleic acid preferentially increased the number of wild type *Ha-ras* mammary tumors, but not the codon 12 mutant *Ha-ras* tumors, in the rat MNU model (10). In chemical carcinogenesis, specific *ras* mutations are induced and are believed to be involved in early stages of tumor development (11–14). Generally, *ras* mutation is considered to be permissive but not sufficient for carcinogenesis. Thus the *ras* genotype was used as a marker in the present study to identify subpopulations of neoplastically transformed cells that might be differentially modulated by CLA intervention.

Materials and methods

Pathogen-free female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories at 45 days of age. They were fed a 20% corn oil diet (6) and were intubated with a single dose of 10 mg of DMBA at 50 days of age for the induction of mammary tumors. Supplementation of CLA (Nu-Chek, Elysian, MN) at 1% in the diet was started 4 days after carcinogen administration. A total of 90 rats were given CLA and were divided equally

Table I. Time course of wild-type and mutant *ras* mammary tumor appearance in control and CLA-supplemented rats^a

Treatment	<i>ras</i> Genotype	Total (%)
Control	Wild-type	49 (84%)
	Mutant	9 (16%)
4 weeks-CLA	Wild-type	49 (92%)
	Mutant	4 (8%)
8 weeks-CLA	Wild-type	43 (86%)
	Mutant	7 (14%)
Continuous-CLA	Wild-type	13 (81%)
	Mutant	3 (19%)

^aThese tumors were harvested from the mammary carcinogenesis experiment described in Figure 1.

into three groups according to the length of CLA treatment: 4 weeks, 8 weeks or continuously until the end of the experiment. Control rats ($n = 30$) were not given CLA at any time during the study.

Animals were palpated weekly for mammary tumors; the time of appearance and location of tumors in the mammary gland were recorded. The experiment was terminated 20 weeks after DMBA. By that time, the development of palpable tumors had plateaued for several weeks across all groups. Only histologically confirmed adenocarcinomas were reported in the results. Tumor incidences at the final time point were compared by chi squared analysis, and the total tumor yield between the control and CLA-treated groups was compared by frequency distribution analysis as described previously (15).

A total of 177 mammary adenocarcinomas were harvested from the above carcinogenesis bioassay. They were individually identified after excision so that each one could be tracked to its time of appearance in a particular rat. All 177 paraffin block-embedded tumors were analyzed for codon 61 *ras* mutation (CAA→CTA) by a modification of the polymerase chain reaction-generated restriction fragment length polymorphism (PCR/RFLP) method as described by Kumar and Barbacid (16). Two 5- μ sections were prepared side-by-side from the same paraffin block, one mounted on a plastic slide, the other on a glass slide, which was subsequently stained with hematoxylin and eosin for the identification of tumor cell foci under the microscope. The exact same area of interest was matched on the plastic slide and was then cut out for DNA extraction (17). The primers used for PCR amplification were 5'-GAGACGTGTTACTGGACATCTT-3' and 5'-GTGTTGTTGATGGCAAA-TACACAGAGG-3' (synthesized by Integrated DNA Technologies, Coralville, IA), which yielded a 116 bp PCR product (18,19). The PCR reaction mixture contained 5 μ l of DNA extract, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 15 μ M deoxynucleotide triphosphate, 1 μ Ci of α -[³²P]dCTP, 0.1 μ M upstream and downstream primers, and 0.5 units AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT). For each batch of PCR reaction, PCR-grade H₂O was used as a blank, DNA from a tumor bearing *Ha-ras* codon 61 mutation as a positive control, and DNA from normal mammary gland as a negative control. Amplification was carried out for 40 cycles at: 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min using a GeneAmp PCR system 9600 (Perkin-Elmer). The codon 61 A→T mutation introduces a Xba I restriction site into the 116 bp PCR product, which upon digestion, generates two fragments of 80 and 36 bp that are diagnostic for the mutation. In contrast, the PCR product of the normal gene contains a sequence that is not susceptible to digestion by Xba I. The digested materials were separated by electrophoresis on a 6% polyacrylamide gel, and detected by autoradiography on X-ray film.

To study the kinetics of CLA retention in the mammary gland, a two-part experiment was conducted to examine (i) the rate of increase of tissue CLA following the start of CLA feeding, and (ii) the rate of disappearance of tissue CLA following CLA withdrawal. For the first part, 60-day-old rats (age-matched to those in the above carcinogenesis experiment but not given DMBA) were fed a 1% CLA diet and were killed at 1, 2, 4, 6 or 8 weeks later. For the second part, rats were fed a 1% CLA diet for 8 weeks, the treatment was discontinued and necropsy was timed at 1, 2, 4 or 6 weeks after CLA withdrawal. Total lipid was extracted from the mammary gland by chloroform/methanol. The separation of neutral lipids and phospholipids was achieved with the use of a Sep-Pak silica cartridge as described in an earlier publication (5). Gas chromatographic analysis of the CLA methyl ester was determined by the method reported previously by Chin et al. (1).

Results

Figure 1 shows the time course of mammary tumor development in control rats or rats fed CLA for various lengths of

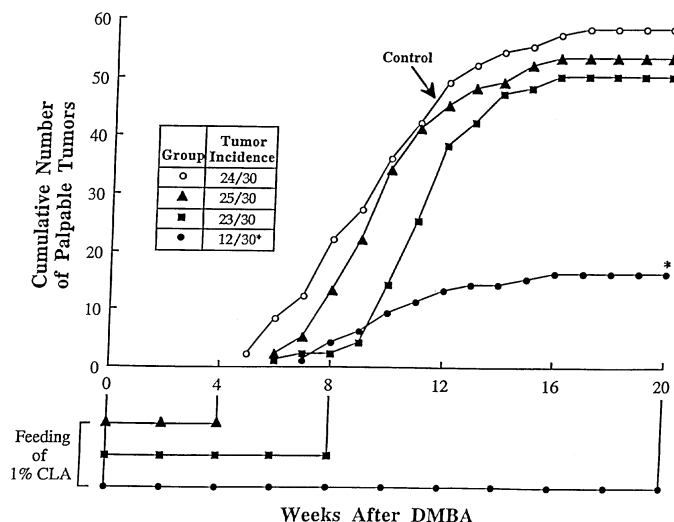


Fig. 1. Effect of interrupted versus continuous CLA feeding after DMBA administration on mammary carcinogenesis. The duration of CLA feeding in the three supplemented groups is indicated along the x-axis time line by the filled symbols, which match the time course of mammary tumor development on the main body of the diagram. Control group without CLA supplementation is represented by the open circle. The asterisk denotes statistically significant difference ($P < 0.05$) from the control data.

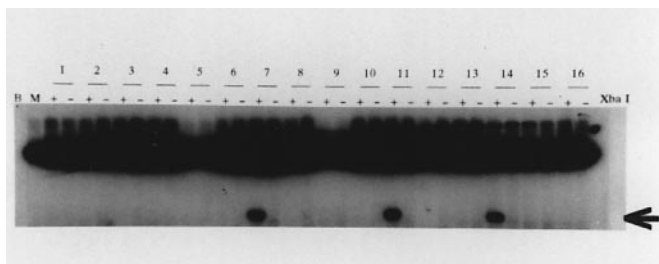


Fig. 2. Detection of *Ha-ras* codon 61 CAA→CTA mutation by PCR/RFLP method. The mutation produces a Xba I site in amplified 116-bp DNA fragment. Upon separation of the Xba I-digested product in 6% polyacrylamide gel, the presence of a 80-bp band (arrow) serves as a diagnostic marker for the mutation. PCR products were labeled with tracer amount of α -[³²P]dCTP and detected by autoradiography. Lane B, blank without template DNA; lane M, normal mammary gland DNA as a negative control; lane 1-16, mammary adenocarcinomas DNA. + and -, 5 μ l of PCR product treated with or without 5 units of Xba I, respectively.

time. It can be seen that short-term feeding of CLA for only 4 or 8 weeks after DMBA administration was not effective in tumor inhibition. In the 8 week-CLA treatment group, the time course curve was shifted slightly to the right, suggesting a delay of about 2 to 3 weeks in the appearance of tumors. However, as soon as CLA was withdrawn, the rate of tumor appearance resumed at a rapid pace. At the time of necropsy, the difference in tumor occurrence between the control group and the 8 week-CLA treatment group was not statistically significant. In contrast, marked cancer protection, as judged by a 50% reduction in tumor incidence and a 70% reduction in the total number of tumors, was observed in rats that were given CLA for the entire duration of the study.

Figure 2 shows some representative electrophoresis autoradiograms of Xba I digested PCR products from tumors with either the wild-type or codon 61 mutant *ras* gene. The arrow in the diagram indicates the presence of a 80-bp band, which is diagnostic for the mutation. Table I summarizes the frequency distribution of both wild-type and mutant *ras* mammary

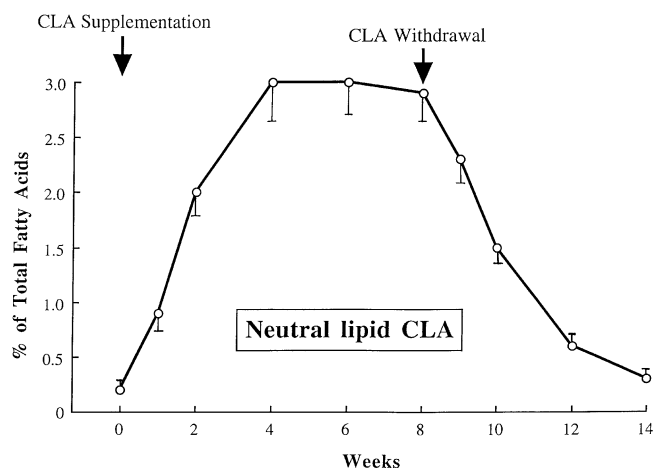


Fig. 3. The kinetics of CLA retention in neutral lipids of mammary gland following CLA supplementation and withdrawal. The results are expressed as the percentage of total fatty acids, mean \pm SE ($n = 6$).

carcinomas from the above experiment. In the control group, 16% of the tumors expressed the mutant *ras* gene. Continuous feeding of CLA reduced the total number of carcinomas by 70%, but was found to suppress approximately the same proportion of wild-type and mutant *ras* carcinomas in comparison with the control group. Short-term treatment with CLA for 4 or 8 weeks did not decrease significantly the total number of carcinomas, nor did it alter markedly the distribution of carcinomas carrying either the wild-type or mutant *ras* gene. Overall, there was no unusual pattern in the time of appearance of the *ras* mutant tumors due to CLA intervention (data not shown). Thus our results indicate that CLA inhibited carcinogenesis irrespective of the presence or absence of the codon 61 *ras* mutation.

Figure 3 shows the rates of CLA accumulation and disappearance in the neutral lipid fraction of the mammary tissue following CLA administration and withdrawal. As the results indicate, the incorporation was rapid once CLA was added to the animals' diets. The level reached ~70% of maximum after 2 weeks of feeding, and plateaued after 4 weeks. At the peak, CLA was present at roughly 3% of total fatty acids in the neutral lipid fraction. In this experiment, some animals were given CLA for 8 weeks. The regimen was stopped, and the decrease in CLA concentration was then plotted in the same composite diagram. Figure 3 shows that as soon as CLA was discontinued, the rate of disappearance from the mammary tissue was equally fast, with a return to basal value in about 4 weeks.

Figure 4 shows the increases and decreases of mammary gland phospholipid CLA from the same experiment. It should be noted that during CLA supplementation, the concentration of CLA in phospholipids (expressed as percent of total fatty acids) was, on the average, an order of magnitude lower than the concentration in neutral lipids. Interestingly, the rate of change of phospholipid CLA in either the upswing or downswing of the exposure/withdrawal curve was slower compared with that observed with neutral lipid CLA. After the start of CLA feeding, the maximum level in phospholipids was not attained until about 6 to 8 weeks later. Similarly, a diminished but still detectable amount of CLA was present by 6 weeks subsequent to the removal of CLA from the diet.

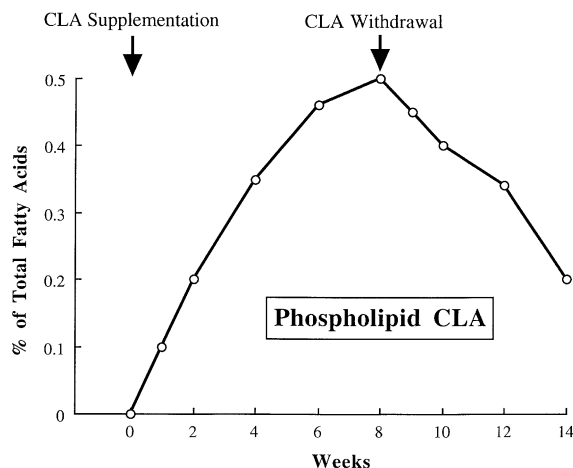


Fig. 4. The kinetics of CLA retention in phospholipids of mammary gland following CLA supplementation and withdrawal.

Discussion

The present study confirms our previous report that a continuous supply of CLA is necessary for maximum tumor inhibition in the post-initiation phase of mammary carcinogenesis. As pointed out in the Introduction, the first experiment was done in MNU-treated rats fed a 5% corn oil diet (9), whereas the repeat experiment described here was carried out in DMBA-treated rats fed a 20% corn oil diet. Thus this characteristic of CLA in chemoprevention is apparently not dependent on specific genomic mutation induced at the time of initiation or the availability of linoleic acid fed to the animals during tumor progression. It might be instructive to contrast the effects of CLA and linoleic acid at this point. Our study here indicated that CLA inhibits mammary carcinogenesis irrespective of the presence or absence of *ras* mutation. Linoleic acid, on the other hand, has been demonstrated to promote selectively the development of the wild type *ras* tumors, but not the mutant *ras* tumors, in MNU-treated rats (10). Recent data also suggested that the response to CLA is unlikely to be due to a displacement of linoleic acid in the mammary tissue (20). Collectively, the above information provides supportive evidence that these two fatty acids may have distinctive mechanisms in the modulation of mammary carcinogenesis.

Mutations of the *ras* gene have been reported to occur in a target organ- and chemical carcinogen-specific manner in a number of experimental models (21). Zarbl *et al.* (22) have previously described that in MNU-induced mammary tumors, GGA \rightarrow GAA mutation in codon 12 of the *Ha-ras* proto-oncogene is a common event. The mutation probably results from methylation of guanine by diazomethane, a spontaneous decomposition product of MNU. In contrast, these same investigators found that only 21% (three out of 14) of DMBA-induced mammary tumors express a CAA \rightarrow CTA mutation in codon 61 of the *Ha-ras* gene (22). It has been proposed that the A \rightarrow T transversion is probably due to the affinity of the DMBA diol epoxide to the adenine residue as well as to the sequence selectivity in binding of the metabolite to the *Ha-ras* DNA (23,24). To our knowledge, there has been one other study examining *Ha-ras* codon 61 mutation in the DMBA model. Interestingly, Waldmann *et al.* (25) did not find such a mutation in a total of 50 tumors. The relatively low incidence of *Ha-ras* codon 61 mutation in our study is similar to that reported by Zarbl *et al.* (22). However, it should be noted that

our analysis was performed on a much larger sample size. In any case, the data in Table I clearly indicate that DMBA-initiated cells, with or without a *Ha-ras* codon 61 mutation, are equally sensitive to the inhibitory activity of CLA.

Recent studies by Banni *et al.* (26) have shown that in rats fed only 0.04% CLA in the diet for 1 week, conjugated diene-C18:3 and -C20:3 were recovered in the liver. Thus it appears that desaturation and elongation of CLA can occur *in vivo* while maintaining the conjugated diene structure. The presence of a conjugated diene-C20:4 metabolite could compete with arachidonic acid for the cyclo-oxygenase and lipo-oxygenase enzymes, thereby altering the biosynthesis of prostaglandins, thromboxanes and leucotrienes. These downstream products of arachidonic acid have been implicated by many investigators to be associated with promotion of carcinogenesis (27–32). By acting as a precursor to conjugated diene-C20:4, CLA could potentially play the role of a metabolic modulator in this process. Thus it becomes imperative to determine if conjugated diene-C20:4 is found in the mammary gland and if it is compartmentalized in a specific lipid fraction.

From our CLA analytical data, it is tempting to postulate that neutral lipid CLA may be a better indicator of protection than phospholipid CLA. Neutral lipid is far more plentiful than phospholipid in the mammary gland (see our previous work quoted in Reference 6). The larger pool of CLA in the former fraction may be more responsive to dietary intake because it serves as a depot for fatty acids that are not immediately utilized. Furthermore, the rate of decay of neutral lipid CLA following CLA withdrawal (Figure 3) seems to match more closely the rate of emergence of new tumors (refer to 4 week- or 8 week-CLA groups in Figure 1).

At first glance, the data on the changes in tissue concentration of CLA (Figures 3 and 4) appear to provide a reasonable explanation of why an uninterrupted supply of CLA is necessary to achieve tumor inhibition. As long as there is an abundant source of CLA present in the target organ, tumor appearance will be blocked or delayed. However, one must not lose sight of the possibility that CLA or a metabolite may induce an effect that is independent of its accumulation in mammary lipids. Future research will be aimed at delineating: (i) whether neutral lipid and phospholipid CLA levels simply represent indicators of CLA exposure; (ii) whether they serve as a local supply of CLA for further metabolism; and (iii) whether different cellular compartments of the mammary gland are involved in the accumulation and metabolism of CLA that ultimately leads to cancer prevention.

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