Morphological and Biochemical Status of the Mammary Gland as Influenced by Conjugated Linoleic Acid: Implication for a Reduction in Mammary Cancer Risk

Henry Thompson, Zongjian Zhu, Sebastiano Banni, Kathleen Darcy, Tami Loftus, and Clement Ip

Division of Laboratory Research, AMC Cancer Research Center, Denver, Colorado 80248; [H. T., Z. Z.]; Dipartimento di Biologia Sperimentale, Sezione di Patologia Sperimentale, Università degli Studi di Cagliari, 09124 Cagliari, Italy [S. B.]; and Department of Experimental Therapeutics, Grace Cancer Drug Center [K. D.], and Department of Surgical Oncology [T. L., C. 1.], Roswell Park Cancer Institute, Buffalo, New York 14263

ABSTRACT

Previous research showed that treatment with conjugated linoleic acid (CLA) during the period of active mammary gland morphogenesis was sufficient to confer a lasting protection against subsequent mammary tumorogenesis induced by methylnitrosourea. The present study was designed to characterize certain morphological and biochemical changes of the mammary gland that might potentially render it less susceptible to cancer induction. Female Sprague Dawley rats were fed a 1% CLA diet from weaning until about 50 days of age. The mammary gland parameters under investigation included (a) the deposition of neutral lipid, (b) the identification and quantification of CLA and its metabolites, (c) the density of the epithelium, and (d) the proliferative activity of various structural components. Our results showed that CLA treatment did not affect total fat deposition in the mammary tissue nor the extent of epithelial invasion into the surrounding fat pad but was able to cause a 20% reduction in the density of the ductal-lobular tree as determined by digitized image analysis of the whole mounts. This was accompanied by a suppression of bromodeoxyuridine labeling in the terminal end buds and lobuloalveolar buds. The recovery of desaturase and elongation products of CLA in the mammary gland confirmed our prior suggestion that the metabolism of CLA might be critical to risk modulation. The significance of the above findings was investigated in a mammary carcinogenesis bioassay with the use of the dimethylbenz[a]anthracene model. When CLA was started at weaning and continued for 6 months until the end of the experiment, this schedule of supplementation produced essentially the same magnitude of mammary tumor inhibition in the dimethylbenz[a]anthracene model as that produced by 1 month of CLA feeding from weaning. The observation is consistent with the hypothesis that exposure to CLA during the time of mammary gland maturation may modify the developmental potential of a subset of target cells that are normally susceptible to carcinogen-induced transformation.

INTRODUCTION

Past research showed that CLA has powerful cancer protective activity in a number of animal tumor models (1—6). With respect to mammary carcinogenesis in the rat, dietary supplementation of CLA has been reported to exert a unique inhibitory effect that is not commonly shared by many anticancer agents. The recent work of Ip and coworkers (7) demonstrated that CLA exposure limited to the period of active mammary gland development was sufficient to confer a lasting protection against subsequent chemically induced tumorigenesis in the target organ. In that experiment, CLA was fed to the animals between 21 days (weaning) and 55 days of age, and a single dose of MNU was administered for mammary tumor induction at day 56. No CLA was provided in the diet after carcinogen treatment. The above observation has a very significant implication for cancer prevention and is the subject of additional research reported herein.

The present study was designed to examine certain morphological and biochemical changes of the mammary gland after 1 month of CLA supplementation starting from weaning. Specifically, our objective was to define the degree of morphological development of the mammary gland as well as alteration in its biochemical constituents that might potentially render it less susceptible to cancer risk. The end points under investigation included (a) the total amount of lipid in the mammary gland, (b) the identification and quantification of CLA and its metabolites in the mammary tissue, (c) the density of the mammary epithelium and the area of the mammary fat pad occupied by the mammary tree, and (d) the proliferative activity of various mammary structural components.

Different carcinogens are known to cause specific mutations that may contribute to the process of oncogenesis (8). As indicated earlier, our initial study regarding the protective effect of CLA following short-term supplementation (instituted at an early age and prior to carcinogen treatment) was done using the MNU model. To rule out that this phenomenon of risk reduction is not an occurrence that is only characteristic of MNU-induced oncomutations, we repeated the tumor experiment with the DMBA model in the study reported here. Additionally, we also evaluated the relative efficacy of the timing of CLA supplementation by comparing the magnitude of mammary cancer inhibition in rats that were fed CLA from weaning to 50 days of age versus those that were given CLA from weaning to the end of the experiment, i.e., including the entire period of tumor promotion and progression. This kind of information is important not only for formulating prevention strategies but also for targeting future research directions.

MATERIALS AND METHODS

Animals and CLA Supplementation. Pathogen-free female Sprague Dawley rats were purchased from Charles River Breeding Laboratories at weaning. They were fed the basal AIN-76A diet with or without supplementation with 1% CLA (Nu-Chek, Elysian, MN). For the studies that were designed to examine the morphological and biochemical changes of the mammary gland, the animals were sacrificed after 1 month on either the control or the CLA diet. The number of rats used in each type of analysis is indicated in the "Results" section. For the mammary carcinogenesis experiment, a total of 120 rats were divided equally into four groups according to the following dietary treatment: group A, control diet from weaning to termination of the experiment (see below); group B, 1% CLA diet from weaning to 50 days of age followed by a switch to the control diet; group C, 1% CLA diet from 55 days of age to termination of the experiment; and group D, 1% CLA diet from weaning to termination of the experiment.

Lipid Extraction. Total lipid was extracted from frozen pulverized mammary tissue by the method of Folch et al. (9). Neutral lipid and phospholipid were separated with the use of a Sep-Pak silica cartridge, as described in an earlier publication (5). The amount of lipid recovered in each of these fractions was measured.

Received 5/30/97; accepted 9/19/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Cancer Institute Grant CA 61763 (to C. L.) and Department of the Army Grant DAMD 17-94-J-A274 (to H. T.).
2 To whom requests for reprints should be addressed, at Department of Surgical Oncology, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, NY 14263.
3 The abbreviations used are: CLA, conjugated linoleic acid; MNU, methylnitrosourea; DMBA, dimethylbenz[a]anthracene; BrdUrd, bromodeoxyuridine.

5067
Quantification of Conjugated Diene Polynsaturated Fatty Acids. For the determination of conjugated diene polynsaturated fatty acids, total lipid extracted from the abdominal-inguinal mammary glands (without separation into neutral lipid and phospholipid) was used as the starting material. Free fatty acids were obtained by a mild saponification procedure as described by Banni et al. (10) and collected in n-hexane. After solvent evaporation, the residue was redissolved in CH\textsubscript{2}Cl\textsubscript{2}/CH\textsubscript{3}COOH (v/v) for injection into the high-performance liquid chromatography 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-\(\mu\)m particle size, 250 \(\times\) 4.6 mm, was used with a mobile phase of CH\textsubscript{2}Cl\textsubscript{2}/H\textsubscript{2}O/CH\textsubscript{3}COOH (70:30:0.12, v/v/v) at a flow rate of 1.5 ml/min. Nonconjugated diene unsaturated fatty acids were detected at 200 nm, and conjugated diene unsaturated fatty acids were detected at 234 nm. Spectra (195–315 nm) of the eluate were obtained every 1.3 s and were stored electronically. 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 high-performance liquid chromatography peaks. Details of the methodology regarding the characterization of conjugated diene fatty acids in both reference and biological samples have recently been published by Banni and coworkers (11).

Preparation of Mammary Gland Whole Mount and Analysis of Epithelial Density. The abdominal-inguinal mammary gland chain was excised in one piece and stretched onto a 75 \(\times\) 50 mm microscope slide. The whole mount was fixed in 10% buffered formalin for 12–18 h and rinsed in distilled water. It was then dehydrated using a series of ethanol solutions (70, 95, and 100%) for 1 h each and cleared with two changes of toluene for 1 h each. The tissue was rehydrated with water and immersed in alum carmine stain for 5–7 days. Once staining was complete, the whole mount was dehydrated using ethanol as described above and cleared with one change of xylene for 2 h. Each whole mount was then placed in a 4 \(\times\) 6-inch heat-sealable pouch and filled with 20 ml of methyl salicylate. Methyl salicylate was chosen as the clearing agent, because its refractive index is very close to that of tissue. This resulted in superior photographic resolution with a clean background. The pouch was left overnight, and on the next day, it was pressed flat to remove excess methyl salicylate and air. All whole mounts were photographed using a Nikon 55 mm camera equipped with a digital camera back. The digitized images were analyzed by scanning densitometry.

All images were presented as an array of pixels. The manipulation of images and all calculations of the parameters were performed on an MPC 200 workstation running the UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT). For each mammary gland chain, the same anatomical region was assessed. This assessment was restricted to the abdominal inguinal mammary gland chain and specifically to the region cephalic to the anterior-most lymph node located in the fourth mammary gland. This lymph node served as an anatomical landmark from which a line perpendicular to the cephalocaudal axis of the animal was projected across the image of the mammary gland. The gland cephalic to this line was cut into image segments, including only the area occupied by the mammary epithelium. The absorbance of each segment and the total area of each segment were then quantified and saved to disk. The density and area of all segments were then summed and averaged. An interactive density thresholding technique was used to select the pixel intensity value (gray value) above which pixels were discriminated from the remainder.

Effect of CLA Feeding on Lipid Content of the Mammary Gland. Recent studies indicated that mice and chickens fed a 0.5% CLA diet showed a 50% reduction in body fat (16). Because deposition of mammary fat is known to regulate epithelial growth in this tissue, we decided to first examine the effect of CLA on the concentration of fat in the mammary gland. Table 1 summarizes the lipid content in the mammary fat pad of rats fed either the control or 1% CLA diet. Both diets were given starting at weaning and continuing for 1 month before the animals were sacrificed. There was no difference in the amounts of total lipid, neutral lipid, and phospholipid that were extractable from the mammary tissue between the two groups. Neutral lipid represented a predominant constituent of total lipid. This is consistent with the fact that triglyceride-containing adipocytes are a major component of the mammary tissue. On the other hand, phospholipid is present at a concentration that is about 100 \(\times\) less. Epithelial cells are the primary source of phospholipid in the mammary gland. The low phospholipid level is reflective of the incomplete differentiation state of the mammary epithelium seen in a nonpregnant and nonlactating animal. The method used for separating the neutral lipid and phospholipid fractions recovered approximately 90% of total lipid. It is possible that more polar lipids might be left on the column.

Assessment of Mammary Epithelial Proliferation by BrdUrd Labeling. Rats were injected i.p. twice a day for 3 consecutive days with 50 mg/kg of BrdUrd dissolved in saline. After excision, the abdominal-inguinal mammary glands were fixed in methacarn, processed in an autotechnicon, and embedded in paraffin blocks. Tissues were cut into 5-\(\mu\)m sections and placed onto 3-aminopropyl-triethoxysilane-prepared slides. The sections were then heat-immobilized, deparaffinized in xylene, rehydrated in descending grades of ethanol, and rinsed in deionized water. The proportion of labeled cells was detected by immunohistochemical staining as described by Eldridge et al. (14). Endogenous peroxidase activity was quenched by incubating in 3% H\textsubscript{2}O\textsubscript{2}. Mouse anti-BrdUrd (Becton Dickinson) was then applied at a dilution of 1:40 for 60 min; this was followed by treatment with biotinylated rabbit antimmouse antibody (DAKO Corp.) and streptavidin horseradish peroxidase. The slides were counterstained with hematoxylin, rinsed, dehydrated, and mounted with Permount. Cells that incorporated BrdUrd were identified by brown granules over the nuclei. One thousand nuclei in each of different mammary compartments (i.e., duct, terminal end bud, and lobuloalveolar bud) were counted, and the number that stained positive was noted.

Mammary Tumor Induction and Determination of Proliferative Activity and Apoptosis in Mammary Tumors. Mammary tumors were induced by the intragastic intubation of 10 mg of DMBA at 50 days of age. Animals were palpated weekly, and the time of appearance and location of tumors in the mammary gland were recorded. The experiment was terminated 21 weeks after DMBA treatment. By that time, the development of palpable tumors had plateaued for several weeks across all groups. Only histologically confirmed adenocarcinomas are reported in “Results.” Tumor incidences at the final time point were compared by \(\chi^2\) analysis, and the total tumor yield between the control and CLA-treated groups was compared by frequency distribution analysis, as described previously (15).

Proliferative activity in the mammary tumors was determined by BrdUrd labeling, as described in an earlier section. Apoptosis in paraffin-embedded tumor sections was evaluated following the procedure supplied with the ApopTag kit, which was purchased from Oncor (Gaithersburg, MD). In brief, the principle of the assay is based on the addition of digoxigenin-labeled nucleotides to the 3'-hydroxyl ends of double- or single-stranded DNA, catalyzed by the terminal deoxynucleotidyl transferase enzyme. An antidigoxigenin antibody conjugated to peroxidase and a chromogenic substrate (diaminobenzidine) are then used to detect incorporated digoxigenin nucleotides in cells.

RESULTS

Effect of CLA Feeding on Lipid Content of the Mammary Gland. Recent studies indicated that mice and chickens fed a 0.5% CLA diet showed a 50% reduction in body fat (16). Because deposition of mammary fat is known to regulate epithelial growth in this tissue, we decided to first examine the effect of CLA on the concentration of fat in the mammary gland. Table 1 summarizes the lipid content in the mammary fat pad of rats fed either the control or 1% CLA diet. Both diets were given starting at weaning and continuing for 1 month before the animals were sacrificed. There was no difference in the amounts of total lipid, neutral lipid, and phospholipid that were extractable from the mammary tissue between the two groups. Neutral lipid represented a predominant constituent of total lipid. This is consistent with the fact that triglyceride-containing adipocytes are a major component of the mammary tissue. On the other hand, phospholipid is present at a concentration that is about 100 \(\times\) less. Epithelial cells are the primary source of phospholipid in the mammary gland. The low phospholipid level is reflective of the incomplete differentiation state of the mammary epithelium seen in a nonpregnant and nonlactating animal. The method used for separating the neutral lipid and phospholipid fractions recovered approximately 90% of total lipid. It is possible that more polar lipids might be left on the column.

Our immediate plan is to investigate this in greater detail and to

Table 1. Lipid content in mammary fat pad of rats fed either control or 1% CLA diet

<table>
<thead>
<tr>
<th>Diet*</th>
<th>Total lipid (mg/g of tissue wet wt)</th>
<th>Neutral lipid (mg/g of tissue wet wt)</th>
<th>Phospholipid (mg/g of tissue wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>266 ± 25</td>
<td>230 ± 20</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>1% CLA</td>
<td>260 ± 16</td>
<td>227 ± 24</td>
<td>2.1 ± 0.3</td>
</tr>
</tbody>
</table>

*Values are expressed as mean ± SE (\(n = 6\)).

Diets were fed from weaning to 50 days of age.
CLA MAMMARY CANCER PREVENTION

Table 2 Conjugated diene polyunsaturated fatty acids (CD-PUFAs) in mammary tissue of rats fed control or 1% CLA diet

<table>
<thead>
<tr>
<th>Diet</th>
<th>CD-PUFA (mmol/mg of lipid)</th>
<th>CD-PUFA (mmol/mg of lipid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>CD 18:2 2.7 ± 0.2</td>
<td>CD 18:2 2.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>CD 18:3 0.4 ± 0.1</td>
<td>CD 18:3 0.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>CD 20:3 0.4 ± 0.1</td>
<td>CD 20:3 0.4 ± 0.1</td>
</tr>
<tr>
<td>1% CLA</td>
<td>CD 18:2 176.9 ± 6.0</td>
<td>CD 18:2 176.9 ± 6.0</td>
</tr>
<tr>
<td></td>
<td>CD 18:3 2.4 ± 0.3</td>
<td>CD 18:3 2.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>CD 20:3 5.6 ± 0.3</td>
<td>CD 20:3 5.6 ± 0.3</td>
</tr>
</tbody>
</table>

*Values are expressed as mean ± SE (n = 6).

† The type of polyunsaturated fatty acid is designated by the length of the carbon chain (18 or 20) and the number of double bonds (2 or 3).

In our previous studies, we suggested that the metabolism of conjugated diene polyunsaturated fatty acids in the mammary gland of CLA-fed rats has not been delineated. Because the composition of individual saturated and unsaturated fatty acids in the mammary tissue of CLA-supplemented rats under various dietary conditions has been described in our earlier publication (5), the results show here will focus only on CLA and its metabolites. Banni et al. (11) have recently published detailed analytical methodologies regarding the identification of various conjugated diene polyunsaturated fatty acids; the same method was used to quantify these derivatives in the mammary gland of control and CLA-fed rats. As shown in Table 2, in addition to CLA (CD 18:2), two other conjugated diene polyunsaturated fatty acids were detected, an 18-carbon triene fatty acid (CD 18:3) and a 20-carbon triene fatty acid (CD 20:3). All three conjugated diene polyunsaturated fatty acids were present at very low levels in the mammary tissue of control rats. Their concentrations were elevated to different degrees upon supplementation with 1% CLA in the diet: 65-fold with CD 18:2, 6-fold with CD 18:3, and 14-fold with CD 20:3. The above finding suggests that CLA could be desaturated further (from a diene to a triene) and elongated (from an 18-carbon to a 20-carbon fatty acid) while still maintaining the conjugated diene structure.

**Effect of CLA on Morphology and Density of the Mammary Epithelium.** It is possible that CLA may reduce mammary cancer risk by directly or indirectly inhibiting the expansion and proliferation of mammary epithelial cells. If this were the case, one might expect to observe a less complex mammary ductal-lobular tree and a reduction in proliferative index associated with the growing epithelium (terminal end buds and/or alveolar buds as opposed to the more stable subtending ducts). Fig. 1 shows representative mammary gland whole mounts from control rats or rats fed 1% CLA. A total of 10 mammary glands were examined from each group. Overall, we could not detect any significant differences in the area of the mammary fat pad occupied by the mammary epithelium as a result of dietary treatment. The density of the mammary epithelium was evaluated by densitometric analysis of the digitized images described in “Materials and Methods.” The data in Table 3 are expressed as absorbance per mm², giving the mean ± SE as well as the 95% confidence interval of the two groups. Our analysis indicated that there was a 21% decrease in the density of the mammary epithelium in the CLA-treated rats. This decrease was statistically significant (P = 0.009).

Table 4 reports the effect of CLA feeding on proliferative activity of the mammary epithelium as measured by BrdUrd labeling. No change was detected in the cells lining the ducts. However, DNA synthesis in the terminal end buds and lobuloalveolar buds was inhibited by about 30% (P < 0.05) as a result of CLA supplementation. The multiple BrdUrd dosing method over a 3-day period (see “Materials and Methods”) increases the sensitivity of the technique by allowing more cells to be labeled and also provides the advantage of being able to mirror steady-state rather than snapshot information. Additionally, this method eviscerates the slight variations in proliferative activity of the mammary epithelial cells due to estrous cycle hormonal surges.

**Effect of Timing of CLA Supplementation on DMBA-induced Mammary Carcinogenesis.** What is the implication of the above morphological and biochemical changes in relation to mammary cancer risk reduction? This question was addressed by the carcinogenesis bioassay. Our primary objective was to determine whether continuous feeding with CLA for 6 months would be more effective in cancer prevention when compared to the 1-month CLA feeding protocol administered during the period of mammary gland morphogenesis. The results of this experiment are shown in Table 5. As indicated in “Materials and Methods,” three different schedules of CLA supplementation were instituted: from weaning to 50 days of age (group B), from 55 days of age to the end of the experiment (group C), and from weaning to the end of the experiment (group D). In all three CLA-supplemented groups as well as in the control group, DMBA was administered to the animals at 50 days of age. At this point, it is important to clarify that our previous work has demonstrated that CLA intake did not affect DMBA binding to mammary DNA (3). Additionally, as will be discussed below, CLA has no effect on phase I and phase II enzymes that are involved in the metabolism of DMBA.

Regardless of whether CLA was given according to the protocol of group B or group C, it reduced the total number of tumors by about 50% (P < 0.05). These observations are consistent with that described in our previous report (5, 7). Interestingly, when CLA was started at weaning and continued to the end of the experiment (Group D), this schedule of supplementation produced essentially a magnitude of tumor inhibition (57%) comparable to that seen in groups B or C. In terms of the timing and length of CLA exposure, group D was representative of the sum of groups B and C. Thus, it might have been predicted that the effects at each stage would have been additive; i.e., the magnitude of tumor inhibition in group D would be in the range of 75% [50% inhibition from feeding CLA prior to DMBA, plus an additional 25% (50% of 50%) inhibition from feeding CLA after DMBA]. The fact that the additive effect was not observed suggests that different mechanisms may be operative depending on whether CLA exposure is coincidental with the period of active mammary gland morphogenesis and development (group B) or occurs during the period of tumor progression after the mature gland is exposed to a carcinogen (group C).

Given the somewhat unexpected result obtained in group D, the above mammary carcinogenesis study was repeated to confirm the reproducibility of the findings. When the data were evaluated at week 15 post-DMBA in the second experiment, the same pattern was found to emerge as that reported in Table 5. Specifically, the magnitude of tumor inhibition was again not statistically different between groups B and D; the total number of palpable tumors was reduced by 45% in group B and 50% in group D. Usually, this type of experiment is maintained for 20 weeks or longer after carcinogen treatment to achieve a plateauing of tumor appearance. In this duplicate experiment, we decided to terminate it at week 15 post-DMBA treatment, so that we could harvest a reasonable number of tumors that would be suitable for the BrdUrd labeling and apoptosis assays. A total of 10 tumors were obtained from group A (control), and 10 tumors were obtained from group D (continuous CLA supplementation). These tumors were chosen based on the criterion that they all showed a fairly uniform growth rate as determined by weekly caliper measurements. Our intention was to avoid the abnormally large tumors (which are frequently necrotic) and the very small tumors (which have a tendency to remain static).

Both the BrdUrd labeling and apoptosis results are reported as a
Table 3 Quantitative analysis of mammary epithelial density in whole mounts of rats fed control or 1% CLA diet

<table>
<thead>
<tr>
<th>Diet</th>
<th>Optical density unit per mm² (Mean ± SE)</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>110 ± 6</td>
<td>96–125</td>
</tr>
<tr>
<td>1% CLA</td>
<td>87 ± 4b</td>
<td>77–97</td>
</tr>
</tbody>
</table>

*The digitized image was taken from photographs as represented in Fig. 1. The data are given as mean ± SE (n = 10) or 95% confidence interval.

Adipocytes are a major and integral cellular component of the rodent mammary gland. They are known to have an important influence on the growth and development of the mammary epithelium. Mammary cells, when transplanted to the gland-free fat pad, will proliferate and expand up to the boundary of the fat pad but not (data not shown). In general, the data support the hypothesis that tumors growing out in rats fed CLA are resistant to the effect of this fatty acid.

**DISCUSSION**

Adipocytes are a major and integral cellular component of the rodent mammary gland. They are known to have an important influence on the growth and development of the mammary epithelium. Mammary cells, when transplanted to the gland-free fat pad, will proliferate and expand up to the boundary of the fat pad but not (data not shown). In general, the data support the hypothesis that tumors growing out in rats fed CLA are resistant to the effect of this fatty acid.

**Table 4 BrdUrd labeling in mammary epithelium of rats fed control or 1% CLA diet**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Mammary compartment (% of cells labeled)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Duct: 15.4 ± 1.5; Terminal end bud: 21.7 ± 2.0; Lobuloalveolar bud: 26.4 ± 2.1</td>
</tr>
<tr>
<td>1% CLA</td>
<td>Duct: 14.6 ± 1.3; Terminal end bud: 15.5 ± 1.3; Lobuloalveolar bud: 18.7 ± 1.9</td>
</tr>
</tbody>
</table>

*There were 12 rats used in each group.

**Fig. 1.** Representative mammary gland whole mounts from a control rat and a rat fed 1% CLA.
CLA MAMMARY CANCER PREVENTION

Table 5 DMBA-induced mammary carcinogenesis in rats fed control or 1% CLA diet

<table>
<thead>
<tr>
<th>Group</th>
<th>Duration of CLA feeding</th>
<th>Tumor incidence</th>
<th>Total No. of tumors</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>26 of 30</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1% CLA From weaning to 50 days of age</td>
<td>17 of 30</td>
<td>42%</td>
<td>49%</td>
</tr>
<tr>
<td>C</td>
<td>1% CLA From 55 days of age to end of experiment</td>
<td>14 of 30</td>
<td>38%</td>
<td>54%</td>
</tr>
<tr>
<td>D</td>
<td>1% CLA From weaning to end of experiment</td>
<td>14 of 30</td>
<td>35%</td>
<td>57%</td>
</tr>
</tbody>
</table>

a DMBA was given to all groups at 50 days of age and the experiment was terminated at 21 weeks post-DMBA.

b Percentage inhibition was calculated using the total tumor number data.

c P < 0.05 compared to the corresponding control group without CLA

Beyond (17, 18). Recent studies from Pariza's laboratory indicated that feeding 0.5% CLA in the diet to mice and chickens reduced their body fat by about 50% (16). The above finding prompted us to investigate whether the deposition of mammary fat in rats would be affected similarly. If CLA were to cause a modest reduction of fat deposition in the mammary gland, such an outcome might lead to less than full development of the mammary epithelium, which might explain the decrease in risk to carcinogenesis. This is clearly not the case, as evidenced by the lack of an effect of CLA on the amount of extractable mammary fat (Table 1). We did, however, find a small but significant reduction in the density of the branching mammary epithelium as a result of CLA feeding (Fig. 1; Table 3). The decrease in lateral branching, as best determined by digitized image analysis, should be distinguished from the unaffected ability of the subtending ducts to invade up to the edge of the surrounding fat pad. Thus, our study suggests that CLA has no apparent effect on fat deposition in the mammary tissue but is able to down-regulate the lateral proliferation of the mammary epithelium via either a direct or an indirect mechanism.

The data in Table 4 show that DNA synthesis (determined by BrdUrd labeling) in the terminal end buds and lobuloalveolar buds was inhibited by CLA. It was therefore gratifying to see that quantitative image analysis of reduced morphogenesis in the mammary gland of CLA-fed rats was confirmed by biochemical measurement of a lower rate of cell proliferation. Terminal end bud cells are the progenitors of lobuloalveolar bud cells. In a 50-day-old rat, most of the terminal end bud cells have already been differentiated to lobuloalveolar bud cells (19). Nonetheless, the former structures are the primary target sites for the induction of mammary carcinomas by chemicals. The lower rate of proliferation in the terminal end bud cells could explain in part the decreased risk to chemical initiation of carcinogenesis.

At this point, it is instructive to contrast the differential effects of CLA on DNA synthesis in normal mammary cells (Table 4) versus mammary tumor cells (Table 6). One interpretation is that CLA may decrease the turnover of normal cells (e.g., terminal end bud cells and lobuloalveolar cells) and inhibit the clonal expansion of early transformed cells (Table 5, group C) but does not affect the proliferative rate of frank carcinoma cells (Table 6). The last part of the conclusion is logical, because the mere appearance of a tumor in a CLA-treated animal implies that the transformed cells in this tumor have already escaped the suppressive effect of CLA. On the other hand, it should be noted that CLA is able to suppress the growth of a human breast cancer line (MDA-MB-468) transplanted in SCID mice, as reported in a recent study (20). Whether this response is unique to the particular cell line grafted to a severely immunodeficient host remains to be investigated. The low frequency of apoptotic cells in the mammary tumors (Table 6) is not unexpected, because the methodology only provides a freeze-frame picture at a given point in time. Additional research is needed to elucidate whether early transformed cells are more sensitive than frank carcinoma cells to CLA-mediated changes in proliferation and apoptosis.

Previous studies have suggested that the metabolism of CLA may be critical in expressing its anticancer activity (21, 22). For example, the conversion of CLA to other related conjugated diene-polyunsaturated fatty acids has a number of potential implications that are relevant to modulation of carcinogenesis. This particular aspect has been discussed in our recent publications (21, 22). Thus, the recovery of desaturation and elongation products of CLA in the mammary gland represents a significant step in this direction. Although CD 18:3 and CD 20:3 were found to be much higher in CLA-fed rats, no CD 20:4 was detected. Suffice it to note that the above analysis was done by using total extractable lipid, which consisted predominantly of neutral lipid because of the contribution from adipocytes. Arachidonic acid is incorporated generally in phospholipid rather than neutral lipid. We are planning to isolate mammary epithelial cells so that a pure phospholipid fraction (free of neutral lipid) can be generated for the analysis of CD 20:4.

Future studies will also focus on the distribution of these conjugated diene polyunsaturated fatty acids in different tissues and blood, as well as in mammary tumors.

The precarcinogen protective effect of CLA (see group B in Table 5) was described initially in rats treated with MNU (7). By using DMBA in the present experiment, we were able to confirm the universality of this response, suggesting that the reduced risk conferred to the animals at an early age is not dependent on carcinogen-specific oncomutations. It is unlikely that CLA achieves this effect by modulating the metabolism of DMBA. As reported previously, CLA does not affect DMBA binding to mammary DNA (3). This observation is consistent with the lack of an effect of CLA on phase I P450 enzymes (1A1, 1A2, 2B1, 2E1, and 3A4),4 and phase II detoxifying enzymes (23). Of particular interest is the finding that short-term supplementation with CLA prior to DMBA (Table 5, group B) is almost as efficacious as the continuous supplementation protocol (Table 5, group D). One possible reason for this is that exposure to CLA during the time of mammary gland maturation might modify the development of a subset of target cells, such that only CLA-resistant mammary epithelial cells were "available" for carcinogen targeting. This hypothesis could account for the reduced cancer risk of group B and the absence of additional protection seen in group D when CLA was continued after carcinogen treatment. In contrast, both the CLA-sensitive and -insensitive subsets of target cells were present in group C at the time of carcinogen administration. Consequently, the feeding of CLA after carcinogen administration was able to suppress the clonal expansion of those transformed cells that originated from the CLA-sensitive progenitors. This interpretation is supported by the

Table 6 BrdUrd labeling and apoptosis in mammary tumors from rats fed either control or 1% CLA diet

<table>
<thead>
<tr>
<th>Diet</th>
<th>Duration of CLA feeding</th>
<th>% positive cells*</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BrdUrd label</td>
<td>Apoptosis</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>From weaning to end of experiment</td>
<td>14.2 ± 1.8</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>1% CLA</td>
<td>From weaning to end of experiment</td>
<td>19.2 ± 2.2</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

* Values are expressed as mean ± SE (n = 10).

4 C. Ip, unpublished data.
data in Table 6, which demonstrate that the proliferative rate of tumors from CLA-fed rats was not inhibited when compared to tumors from control rats. Although somewhat simplistic, the above concept offers a unifying hypothesis to interpret the collective results described in this paper, and is a reasonable starting point to investigate in depth the multiple mechanisms involved in the action of CLA in cancer prevention.

ACKNOWLEDGMENTS

We are grateful to Todd Parsons, Rita Pawlak, and John McGinley for their technical assistance.

REFERENCES

Morphological and Biochemical Status of the Mammary Gland as Influenced by Conjugated Linoleic Acid: Implication for a Reduction in Mammary Cancer Risk

Henry Thompson, Zongjian Zhu, Sebastiano Banni, et al.

Cancer Res 1997;57:5067-5072.