The \(\text{t}10,\text{c}12\) isomer of conjugated linoleic acid stimulates mammary tumorigenesis in transgenic mice over-expressing \(\text{erbB}2\) in the mammary epithelium

Margot M.Ip \(^1\), Sibel O.McGee, Patricia A.Masso-Welch \(^2\), Clement Ip \(^1\), Xiaojing Meng, Lihui Ou and Suzanne F.Shoemaker

Department of Pharmacology and Therapeutics and \(^1\)Department of Chemoprevention, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA and \(^2\)Biotechnical and Clinical Laboratory Sciences, University at Buffalo, Buffalo, NY, USA

\(^1\)To whom correspondence should be addressed. Tel: +1 716 845 2356; Fax: +1 716 845 5865; Email: margot.ip@roswellpark.org

Conjugated linoleic acid (CLA), a family of isomers of octadecadienoic acid, inhibits rat mammary carcinogenesis, angiogenesis, and lung metastasis from a transplantable mammary tumor. \(\text{c}9,\text{t}11\)-CLA, the predominant isomer in dairy products, and \(\text{t}10,\text{c}12\)-CLA, a component of CLA supplements, are equally effective. The objective of the current studies was to test the efficacy of these two CLA isomers in a clinically relevant breast cancer model. Transgenic mice over-expressing \(\text{erbB}2\) in the mammary epithelium were fed control or 0.5% CLA-supplemented diets continuously from weaning. Unexpectedly, \(\text{t}10,\text{c}12\)-CLA stimulated lobular hyperplasia of the mammary epithelium and accelerated mammary tumor development, decreasing median tumor latency to 168 days of age compared with 256 and 270 days in the \(\text{c}9,\text{t}11\)-CLA and control groups, respectively. Metastasis was also increased by \(\text{t}10,\text{c}12\)-CLA, with percentage of tumor-bearing mice with lung metastasis 73, 14 and 31% in the \(\text{t}10,\text{c}12\)-CLA, \(\text{c}9,\text{t}11\)-CLA and control groups, respectively. A second study, in which CLA administration was initiated after puberty, confirmed the stimulatory effect of \(\text{t}10,\text{c}12\)-CLA on mammary tumor development and metastasis. Additionally, \(\text{t}10,\text{c}12\)-CLA, but not \(\text{c}9,\text{t}11\)-CLA, increased the size of the liver, heart, spleen and mammary lymph node. The effects of \(\text{t}10,\text{c}12\)-CLA were not specific to \(\text{erbB}2\) transgenic mice, as \(\text{t}10,\text{c}12\)-CLA supplementation increased proliferation in the mammary epithelium of both wild-type FVB and FVB/\(\text{erbB}2\) mice. Moreover, the number of terminal end buds, the mammary epithelial structures most sensitive to a carcinogenic insult, was increased 30-fold in FVB wild-type mice fed \(\text{t}10,\text{c}12\)-CLA. These data suggest that it would be prudent to avoid CLA supplements containing the \(\text{t}10,\text{c}12\)-CLA isomer. However, even though \(\text{c}9,\text{t}11\)-CLA was not efficacious in the \(\text{erbB}2\) model, its ability to inhibit mammary tumor development in rat models suggests that it may have activity for prevention of some types of breast cancer.

Materials and methods

**Animals and dietary treatments**

FVB/N-Tg (MMTV\(\text{neu}\))\(202\text{Mul/J}\) (15) and FVB/J female mice were obtained from Jackson Laboratories (Bar Harbor, ME). In the first tumorigenesis experiment (CLA from weaning), mice were randomized by weight into three groups of 30 mice per group and placed on a basal AIN-76A diet without or with 0.5% \(\text{c}9,\text{t}11\)-CLA or \(\text{t}10,\text{c}12\)-CLA (16) at 24 days of age. In the second tumorigenesis experiment (CLA after puberty), mice were randomized into three groups of 25 mice per group and placed on one of the above diets at 68–72 days of age. The diets were fed continuously throughout the experiment. Mice were euthanized when a tumor reached a size of 18–20 mm in the larger diameter. Mammary glands and tumors were removed and prepared for whole-mount analysis and/or fixed in formalin. Lungs of the euthanized mice were perfused with \(~1.5\) mL India ink (15% in water) through the trachea. The lungs were then removed, rinsed in water and fixed in Fekete’s solution [70% ethanol (v/v), 3.7% paraformaldehyde (v/v) and 0.75 M acetic acid]. For the experiments to compare proliferation and apoptosis in wild-type and transgenic mice, FVB/J (seven mice per group) or FVB/N-Tg (MMTV\(\text{neu}\))\(202\text{Mul/J}\) (five mice per group) were fed control or 0.5% CLA-supplemented diets for 10 days starting at 70 days of age. Mice were maintained in microisolator cages in a temperature- and humidity-controlled environment with a 12 h light–dark cycle, and were given food and water ad libitum. Animals were housed in accordance with the standards set by the National Institutes of Health and the Roswell Park Cancer Institute Animal Care and Use Committee.

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Dietary ingredients

The CLA isomers were obtained from Natural ASA (Hovdebygda, Norway) or Larodan Fine Chemicals (Malmö, Sweden); the purity of each isomer was >90%. The other diet ingredients were purchased from the following sources: casein and corn oil, Harlan Teklad (Madison, WI); dextrose, Federal Bakers Supply (Buffalo, NY); AIN-76A vitamin mix, AIN-76 mineral mix, alphacel and dl-methionine, MP Biomedicals (Irvine, CA) and choline bitartrate, Sigma (St Louis, MO).

Preparation of mammary gland whole mounts

Mammary glands four and five were removed in one piece and spread out on slides according to the original anatomical orientation. After air drying on the slide for 10–15 min, the mammary glands were fixed for 15–19 h in Carnoy’s fixative (75% (v/v) ethanol and 25% (v/v) acetic acid), washed in 70% (v/v) ethanol and hydrated in H$_2$O and then stained with 0.2% (w/v) carmine (Sigma Chemical Company) in 5% (w/v) aqueous AlK(SO$_4$)$_2$·12H$_2$O for 18–48 h. Tissues were then dehydrated through 15 min incubations in 70% (v/v), 95% (v/v) and 100% ethanol, defatted in acetone for 1–1.5 h and cleared in xylene for at least 24 h before incubating in Histoclear (National Diagnostics, Atlanta, GA) for 15 min and cover slipping with Permount (Fisher Scientific, Fair Lawn, NJ). Whole mounts were photographed using an Olympus SZ-PT stereoscope or an Olympus BX-40 microscope with an attached Hitachi KP-D50 color digital camera.

Histology, immunohistochemistry and evaluation of metastasis

Paraffin sections of mammary glands and tumors were stained with hematoxylin and eosin (H and E) for evaluation of histology, and immunohistochemistry was performed using 2 or 4 µg/ml rabbit polyclonal erbB2 antibody (#2428, Novus Biologicals, Littleton, CO) or 1 µg/ml polyclonal Ki67 antibody (#500-170) (Novus Biologicals). The ApoTag® Plus Peroxidase In Situ Apoptosis Kit (Chemicon, Temecula, CA) was used on paraffin sections to detect apoptosis. To evaluate metastasis in the lung, the entire lung was sectioned, and mice were scored as either negative or positive for lung metastasis, which is visualized as white nodules on the black ink-stained lungs.

Statistics

Log rank analysis was used to statistically evaluate the Kaplan–Meier curves, with the Holm–Sidak method used to analyze multiple curves. Statistical differences in tumor multiplicity, survival time after development of a palpable tumor, body weight, organ sizes, terminal end bad number and percentage of Ki67- or TUNEL-positive nuclei were tested by one-way analysis of variance, with the Holm–Sidak method used for pairwise multiple comparisons. Differences in lung metastases were tested by chi-square analysis. A $P$ value of $<0.05$ was considered statistically significant. Where shown, error bars are standard error of the mean.

Results

$t_{10,12}$-CLA stimulates mammary tumor development and lung metastasis in erbB2 transgenic mice

The effects of dietary CLA were tested in a model relevant to a significant proportion of breast cancer patients in order to determine if the inhibitory effects of the c9,t11 and t10,c12-CLA isomers could be translated to this group of women. Mice over-expressing wild-type erbB2 in the mammary epithelium (hereby termed ‘erbB2 transgenic mice’) were fed control or CLA-containing diets continuously from 24 days of age. Dietary c9,t11-CLA had no significant effect on the rate at which mammary tumors developed in erbB2 transgenic mice (Figure 1A), or on the number of tumors per mouse (data not shown) or on the metastasis to the lung (Figure 1B; $P = 0.21$, not significant). Unexpectedly, however, dietary t10,c12-CLA markedly accelerated mammary tumor development. Latency, or the age at which 50% of the mice had developed a tumor, was only 168 days in the t10,c12-CLA group compared with 270 days in the control (Figure 1A, $P < 0.001$). Latency in the c9,t11-CLA group was 256 days (not significant, $P = 0.40$ compared with control). Tumor multiplicity was not altered by t10,c12-CLA (data not shown); however, lung metastasis was dramatically increased (Figure 1B, $P = 0.003$). This increased metastasis was not a result of an alteration in primary tumor burden, since all mice were euthanized with a similar tumor size.

In spite of the earlier time of tumor appearance and increased metastasis in the t10,c12-CLA group, the tumors did not grow more rapidly in this group. To evaluate this, we determined the survival time of tumor-bearing mice with metastasis. The control and the c9,t11-CLA groups are each statistically different from the t10,c12-CLA group; there is no statistically significant difference between the control and c9,t11-CLA groups. (B) Percentage of tumor-bearing mice with lung metastasis. The numbers above each bar indicate the number of tumor-bearing mice (denominator) with lung metastasis (numerator) in each group. The control and c9,t11-CLA groups are each statistically different from the t10,c12-CLA group. (C) Body weights of erbB2 transgenic mice in the three dietary groups. Each point represents the mean ± SEM.

Fig. 1. Mammary tumorigenesis, lung metastasis and body weight of erbB2 transgenic mice fed control, c9,t11-CLA- or t10,c12-CLA-supplemented diets from 24 days of age until being killed. (A) Kaplan–Meier plot showing percentage of tumor-free mice in each group at various ages. Of the initial 30 mice in each group, one mouse in the control group and one in the c9,t11-CLA group were killed early due to causes unrelated to the experimental protocol. These mice are not included in the figure. The remaining mice in the control group (29 mice) and all mice in the t10,c12-CLA group (30 mice) were killed with tumor. One mouse in the c9,t11-CLA group was killed without tumor at the end of the experiment; 28 mice in the c9,t11-CLA group were killed with tumor. The control and the c9,t11-CLA groups are each statistically different from the t10,c12-CLA group. (B) Percentage of tumor-bearing mice with lung metastasis. The numbers above each bar indicate the number of tumor-bearing mice (denominator) with lung metastasis (numerator) in each group. The control and c9,t11-CLA groups are each statistically different from the t10,c12-CLA group. (C) Body weights of erbB2 transgenic mice in the three dietary groups. Each point represents the mean ± SEM.
of mice in each group, defined as the time between detection of a palpable tumor (2–3 mm) and the time of kill when a tumor reached 18–20 mm in the longer diameter. Mice fed the control, c9,t11- or t10,c12-CLA-supplemented diets were killed 51.7 ± 3.8, 48.6 ± 3.5 and 63.7 ± 4.2 days, respectively, after initial detection of a palpable, measurable tumor. This modest increase in survival time once a palpable tumor had been detected (control versus t10,c12-CLA, \( P = 0.029 \)) suggests that t10,c12-CLA may have been exerting a slight inhibitory effect on tumor growth during this time period.

The increased tumor development in the t10,c12-CLA-fed mice was not secondary to an increase in body weight since mice in this group actually weighed less than those in the control group (Figure 1C, \( P < 0.01 \)). Food intake in the t10,c12-CLA-fed group was slightly, but not significantly, increased compared with the other two groups (data not shown).

t10,c12-CLA alters development of the mammary epithelium but does not alter expression or localization of erbB2

In association with the accelerated tumor development, t10,c12-CLA had a marked effect on the mammary epithelium. Most striking, as seen from the mammary whole mounts (Figure 2A–D), was a precocious alveolar-like branching and hyperplasia that was continuous along the whole ductal network in this dietary group. This contrasts with the more normal-appearing ductal network in the control and c9,t11-CLA groups of erbB2 transgenic mice. Additionally, in the t10,c12-CLA group, the ducts were very dilated (Figure 2D) compared with the control or c9,t11-CLA groups (Figure 2B and C), and the epithelium did not grow to the boundary of the mammary fat pad (Figure 2A; the numbers under the whole mounts refer to the length of the epithelium from the lymph node). It can also be seen that mammary glands from t10,c12-CLA-fed mice were much smaller (Figure 2A), and on viewing the H- and E-stained cross-sections (Figure 3A), a markedly reduced adipocyte content [both brown and white adipose tissue] and an increase in fibrocellular stroma (arrows) were noted. No differences were apparent between the control and c9,t11-CLA groups (Figures 2 and 3). Finally, as observed in Figure 2A, the intra-mammary lymph node was significantly larger in the mammary glands from mice fed the t10,c12-CLA-supplemented diet; the lengths were 2.27 ± 0.06, 2.19 ± 0.05 and 3.74 ± 0.13 mm (\( n = 16–17 \)) in the control, c9,t11-CLA and t10,c12-CLA groups, respectively. Together with the increased size of the spleen in the t10,c12-CLA group (see below), this suggests that this isomer may cause both systemic and local alterations in lymphoid function.

We considered the possibility that the increased tumorigenesis in mice fed the diet supplemented with t10,c12-CLA was a result of an increased expression of erbB2. However, immunohistochemical detection of erbB2 in mammary glands from each of the groups demonstrated no changes in expression or subcellular localization of erbB2 (Figure 3B). Most cells within the mammary ducts and lobules expressed erbB2 along the lateral, basal and apical membranes, and no differences in staining intensity were noted. Compared with ducts and lobular ductules, erbB2 expression in tumors was highly heterogeneous and not significantly different among the groups (data not shown). These data suggest that the effects of t10,c12-CLA were not a result of changes in erbB2.

t10,c12-CLA increased the size of the liver, heart and spleen

In addition to stimulating mammary tumorigenesis, absolute as well as relative weights of the liver, heart and spleen were significantly increased in mice fed the diet supplemented with t10,c12-CLA (Table I). No differences were noted between the control and c9,t11-CLA groups. When we examined H and E sections of the spleen, an increase in the size of the white pulp was observed in the t10,c12-CLA group, and could account for the majority of the increased spleen size in this group. In particular, the spleens of mice fed t10,c12-CLA showed an enlargement of follicular areas, which was due to an increase in primary follicles rather than germinal centers, which were noticeably lacking. The expansion of the white pulp in the absence of germinal center development, which was abundant in control and c9,t11-CLA-fed mice, suggests that t10,c12-CLA may suppress the development of a secondary immune response in these tumor-bearing mice. Finally, the livers in the t10,c12-CLA group were much lighter in color, and upon H and E staining, liver epithelium was heavily vacuolated in appearance, indicative of fatty liver (data not shown).

Delaying CLA feeding until after puberty does not abrogate the stimulatory effect of t10,c12-CLA on mammary tumorigenesis

To eliminate a potential confounding effect of t10,c12-CLA on the mammary epithelium during pubertal development, a second experiment was performed in which erbB2 transgenic mice were fed control or CLA-supplemented diets starting after puberty at 68–72 days of age. Under these conditions, a statistically significant stimulatory effect of t10,c12-CLA on mammary tumorigenesis and lung metastasis was again seen (Figure 4). Median times of mammary tumor development in this study were 271, 263 and 207 days of age in the control, c9,t11-CLA and t10,c12-CLA groups, respectively (\( P < 0.001 \) for t10,c12-CLA versus control or c9,t11-CLA). Once a palpable tumor was detected, survival times in each of the groups were
fed the diet supplemented with t10,c12-CLA, but not in those fed the control or c9,t11-CLA diets (Figure 5).

Dietary t10,c12-CLA stimulates proliferation of the mammary epithelium of both FVB/erbB2 transgenic and FVB wild-type mice

To evaluate whether erbB2 over-expression was necessary for the stimulatory effect of t10,c12-CLA, a short-term experiment was undertaken in which the effects of CLA on the Ki67 labeling index were compared in the mammary epithelium of FVB/erbB2 transgenic and FVB wild-type mice. Ki67 is a proliferation marker that is expressed throughout the active phases of the cell cycle, but not in G0, and it has been shown to be an independent prognostic indicator in breast cancer (19). Mice were fed the control or CLA-containing diets for 10 days, starting at 70 days of age, the time at which CLA supplementation was initiated in the second mammary tumorigenesis study. The 10 day feeding period was chosen based on our earlier studies demonstrating that t10,c12-CLA-induced changes in the mammary stroma within 3 days (16). Table II shows that t10,c12-CLA significantly increased the Ki67 labeling index in the terminal end buds, ducts and lobules of the mammary epithelium in both FVB wild-type and FVB/erbB2 mice, although the effect was more dramatic in the transgenic mice. Moreover, extensive budding of the epithelium and dilatation of the ducts were observed after only 10 days of feeding the FVB wild-type mice the t10,c12-CLA-supplemented diet, and the number of terminal end buds was markedly increased (Figure 6). Apoptosis was low in both wild-type and transgenic mice, and in the wild-type mice was not significantly altered by either CLA isomer (Table II). A significant increase in apoptosis was observed in the FVB/erbB2 transgenic mice fed the t10,c12-CLA-supplemented diet; however, the percentage of cells undergoing apoptosis in this group was considerably less than those undergoing proliferation (Table II).

Discussion

Dietary CLA, fed as a mixed isomer preparation containing equal amounts of c9,t11-CLA and t10,c12-CLA, as well as the individual isomers, has been shown previously to inhibit carcinogen-induced rat mammary tumorigenesis, as well as the growth and/or metastases of mouse mammary tumors in syngeneic and xenograft models (reviewed in ref. 7). Together, these studies were strongly supportive of the notion that dietary CLA might have clinical application in inhibiting the development and metastatic spread of breast cancer, as well as reducing residual disease. However, results from the current experiments demonstrating that the t10,c12 isomer of CLA increases mammary tumor development and metastasis in mice over-expressing wild-type erbB2 in the mammary epithelium clearly dispel this notion for the t10,c12-CLA isomer. Moreover, our data suggest that the detrimental effect of t10,c12-CLA is independent of the time at which

**Table I. Effect of long-term dietary CLA on mouse organ and body weights**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control diet</th>
<th>c9,t11-CLA diet</th>
<th>t10,c12-CLA diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>35.4 ± 1.0</td>
<td>34.0 ± 1.0</td>
<td>29.1 ± 0.7</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.53 ± 0.04</td>
<td>1.57 ± 0.04</td>
<td>2.59 ± 0.11</td>
</tr>
<tr>
<td>Liver weight (g/100 g body wt)</td>
<td>4.37 ± 0.12</td>
<td>4.66 ± 0.11</td>
<td>8.89 ± 0.30</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>132 ± 4</td>
<td>129 ± 3</td>
<td>177 ± 7</td>
</tr>
<tr>
<td>Heart weight (mg/100 g body wt)</td>
<td>374 ± 11</td>
<td>385 ± 14</td>
<td>615 ± 18</td>
</tr>
<tr>
<td>Spleen weight (mg)</td>
<td>149 ± 9</td>
<td>146 ± 8</td>
<td>252 ± 14</td>
</tr>
<tr>
<td>Spleen weight (mg/100 g body wt)</td>
<td>423 ± 23</td>
<td>437 ± 28</td>
<td>858 ± 35</td>
</tr>
</tbody>
</table>

Mean ± SEM. Means in a row without a common letter are statistically different. N = 28–29, 26–28 and 29–30 for control, c9,t11-CLA and t10,c12-CLA groups, respectively. Tissues were obtained from the transgenic erbB2 mice used in the first tumorigenesis study, in which the control or CLA-supplemented diets were fed from weaning.
supplementation is initiated, since tumorigenesis was accelerated in both the prepubertal and postpubertal experimental protocols, with a mean time of tumor appearance 144 and 137 days, respectively, after the mice were started on the t10,c12-CLA-containing diets. The effect was isomer specific, since c9,t11-CLA did not alter tumor-igenesis or metastasis.

Human breast cancer and erbB2/her2
ErbB2/her2 is naturally expressed in normal breast epithelium of humans (20,21), as well as in rat (22) and mouse (23) mammary glands. Over-expression of erbB2 is observed in 20–30% of human breast cancers, and is associated with a poor prognosis (14,24,25). This increased expression of erbB2 is seen in ductal carcinoma in situ, an early stage of breast cancer development, and interestingly its frequency is higher in ductal carcinoma in situ than in invasive breast cancers (26–30). No over-expression has been noted in hyperplastic or dysplastic lesions (28). Although our experiments examined the effect of diet on the acceleration of mammary tumorigenesis in erbB2 transgenic mice, it is important to note that erbB2 is normally expressed in normal breast epithelium and its over-expression is associated with a poor prognosis.

Fig. 4. Diets supplemented with t10,c12-CLA after puberty, but not with c9,t11-CLA, accelerate mammary tumor development and increase lung metastasis of erbB2 transgenic mice. Diets were initiated at 68–72 days of age, and fed continuously. (A) Kaplan–Meier plot showing percentage of tumor-free mice in each group at various ages. Of the initial 25 mice in each group, one mouse in the control group, four in the c9,t11-CLA group and two in the t10,c12-CLA group were killed early due to causes unrelated to the experimental protocol. These mice are not included in the figure. The remaining mice in the control (24 mice), c9,t11-CLA (21 mice) and t10,c12-CLA (23 mice) groups were killed with tumor. The control and the c9,t11-CLA groups are each significantly different from the t10,c12-CLA group; there is no statistically significant difference between the control and c9,t11-CLA groups. (B) Percentage of tumor-bearing mice with lung metastasis. The numbers above each bar indicate the number of tumor-bearing mice (denominator) with lung metastasis (numerator) in each group. Evaluation of metastasis could not be performed in one control mouse and one t10,c12-CLA mouse. The control and c9,t11-CLA groups are each statistically different from the t10,c12-CLA group.

Fig. 5. Mammary gland whole mounts from erbB2 transgenic mice fed the control, c9,t11-CLA- and t10,c12-CLA-supplemented diets. Diets were initiated at 68–72 days of age, and fed continuously (same experiment as shown in Figure 4). Note the extensive budding and alveolar hyperplasia from the ducts in the t10,c12-CLA group. The arrows point to vacuole-like structures within the ducts from the t10,c12-CLA group, and the arrowhead to an alveolar lumen. Photos were taken from mammary whole mounts using the ×1 (left side) or ×4 (right side) microscope objectives.
of dietary CLA in a model in which the majority of the mammary epithelial cells (MECs) over-express erbB2, and as a result could overestimate the effect of t10,c12-CLA in human breast cancer, the high frequency of erbB2 over-expression (40–77%) in ductal carcinoma in situ (26–30), together with the high incidence of breast cancer, suggests that a large population of women with clinical or preclinical disease may be susceptible to the deleterious effect of t10,c12-CLA.

Why does t10,c12-CLA stimulate mammary carcinogenesis in erbB2 transgenic mice?

Our observation that t10,c12-CLA accelerates mammary tumor development and increases metastasis, and yet does not alter survival time after a palpable tumor is detected, is something of a conundrum, but may reflect the multifaceted activity of this CLA isomer. For example, we demonstrated previously that in wild-type mice, t10,c12-CLA inhibits angiogenesis (as does c9,t11-CLA), concurrent with a decrease in the pro-angiogenic and tumor growth factors vascular endothelial growth factor and leptin (16,31). This could limit tumor expansion. On the other hand, the acceleration of tumor development, as well as the increased metastasis to the lung, suggests that t10,c12-CLA has modified the mammary epithelium and/or its stromal environment, resulting in an increased sensitivity to tumor development. This notion is supported by the dramatically altered morphological appearance of the mammary epithelium and stroma in mice fed the t10,c12-CLA-supplemented diet. Indeed, the extensive epithelial budding, which was continuous along the entire ductal network, suggested that the proliferative capacity of the epithelium was increased at least at early times after the mice received this CLA isomer. In fact, we found this to be the case, as the Ki67 labeling index was increased in the erbB2 transgenic mice after only 10 days of feeding t10,c12-CLA. This increased proliferation could account for the earlier time of tumor appearance in this group.

Unexpectedly, feeding of t10,c12-CLA for only 10 days also stimulated the Ki67 labeling index in the erbB2 transgenic mice after only 10 days of feeding t10,c12-CLA. This increased proliferation could account for the earlier time of tumor appearance in this group.

\[ \text{Fig. 6. Mammary gland whole mounts from FVB wild-type mice fed the control, c9,t11-CLA- and t10,c12-CLA-supplemented diets for 10 days starting at 70 days of age. Extensive budding and dilated ducts can be seen in the t10,c12-CLA group. The numbers in each panel refer to the number of terminal end buds (mean ± SEM of seven mice per group). Each photo in the left panels of the three dietary groups was taken under the ×1 objective. Each photo in the right panels of the three dietary groups was taken under the ×4 objective.} \]
signals resulting from t10,c12-CLA supplementation and erbB2 overexpression may converge downstream to enhance tumor development. The mechanism by which this may occur is currently not known; however, two observations point to fruitful areas of future research. First, the intra-mammary lymph node, which is immediately adjacent to the epithelium, was visibly enlarged, and as a result, there may be changes in cytokine secretion that could impact growth of the epithelium. Second, the fibroblastic stroma surrounding the mammary ductal epithelium in the t10,c12-CLA-supplemented mice is reminiscent of the reactive stroma that surrounds breast tumors, and it is tempting to speculate that it plays an important role in modulating the tumorigenic response of the erbB2-over-expressing epithelium and the proliferative response of wild-type mouse epithelium. In support of this possibility, it is significant that t10,c12-CLA has no readily discernible effect on the rat mammary stroma (33 and P.Masso-Welch and M.M.Ip, unpublished data), and a mixture of CLA isomers containing approximately equal amounts of t10,c12-CLA and c9,t11-CLA inhibited, rather than stimulated rat mammary epithelial proliferation and carcinogenesis (33).

The pregnancy-like increase in lobular–alveolar development of the mammary epithelium in the t10,c12-CLA group of erbB2 mice was unexpected. A possible explanation is that the marked increased in the fibroblastic stroma surrounding the ducts (Figure 3), which we also observed in wild-type CD2F1 mice (16), could result in an enhanced deposition of extracellular matrix, a known stimulator of alveolar differentiation (34). However, since contact with the extracellular matrix is required but not sufficient (35,36) for alveolar development, additional changes in the local or systemic hormonal or growth factor milieu would have to be postulated.

An intriguing consequence of the increased differentiation may be the induction of a MEC population that is functionally equivalent to the pregnancy-induced MEC sub-population remaining in the mammary gland after post-weaning involution of the epithelium (37,38). These parity-induced MECs are pluripotent and capable of self-renewal (38), and importantly, were shown to be the specific targets for mammary tumorigenesis in the mouse MMTV-erbB2 overexpression model (39). The relevance of this concept to human breast cancer should also be considered. Henry et al. (39) have pointed out that transgenic mouse models of the type used in our study may represent a good model for the transient increased risk of breast cancer after a full-term pregnancy. If our data were translatable to the human situation, this could imply that some women consuming t10,c12-CLA during and immediately after a full-term pregnancy might be at an increased risk of breast cancer, especially if they have pre-existing erbB2-over-expressing cells within the epithelium.
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