t10,c12-Conjugated linoleic acid stimulates mammary tumor progression in Her2/ErB2 mice through activation of both proliferative and survival pathways

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The t10,c12 isomer of conjugated linoleic acid (CLA) inhibits rat mammary carcinogenesis, metastasis from a transplantable mouse mammary tumor and angiogenesis; however, it stimulates mammary tumorigenesis in transgenic mice overexpressing ErbB2 in the mammary epithelium (ErbB2 transgenic mice). In the current study, we report that a 4-week supplementation of the diet with 0.5% trans-10, cis-12 conjugated linoleic acid (t10,c12-CLA) stimulated the growth of established ErbB2-overexpressing mammary tumors by 30% and increased the number of new tumors from 11% to 82%. Additionally, when t10,c12-CLA supplementation of ErbB2 transgenic mice was initiated at 21 weeks of age, a time just prior to tumor appearance, overall survival was decreased from 46.4 weeks in the control to 39.0 weeks in the CLA group, and survival after detection of a palpable tumor from 7.5 to 4.6 weeks. Short-term supplementation from 10 to 14 weeks or 21 to 25 weeks of age temporarily accelerated tumor development, but over the long term, there was no significant effect on mammary tumorogenesis. Long term as well as short a 4-week supplementation increased mammary epithelial hyperplasia and lobular development, and altered the mammary stroma; this was reversible in mice returned to the control diet. t10,c12-CLA altered proliferation and apoptosis of the mammary epithelium, although this differed depending on the length of administration and/or the age of the mice. The increased tumor development with t10,c12-CLA was associated with increased phosphorylation of the IGF-I/insulin receptor, as well as increased signaling through the mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase and phosphatidylinositol 3-kinase/Akt pathways; however, neither phospho-ErbB2 nor ErB2 was altered.

Introduction

Conjugated linoleic acid (CLA) refers to a mixture of positional and geometric isomers of linoleic acid with two conjugated double bonds. cis-9, trans-11 conjugated linoleic acid (c9,t11-CLA) is the predominant isomer that is naturally present in dairy products and ruminant meat. CLA is also produced synthetically, and a mixture of trans-10, cis-12 conjugated linoleic acid (t10,c12-CLA) and c9,t11-CLA is currently available as a health supplement. A number of studies have suggested that CLA might be a potential breast cancer chemopreventive agent. For example, each of these CLA isomers, as well as a mixture containing these two isomers, were shown to inhibit the initiation and progression stages of chemically induced rat mammary carcinogenesis (1–4), as well as inhibit metastasis in a syngeneic mouse mammary tumor model (5,6). CLA isomers, particularly t10,c12-CLA, are also efficacious in inhibiting growth and inducing apoptosis.

Abbreviations: CLA, conjugated linoleic acid; c9,t11-CLA, cis-9, trans-11 CLA; ERK, extracellular signal-regulated kinase; H&E, hematoxylin and eosin; IGF-IR, insulin-like growth factor I receptor; MEK, mitogen-activated protein kinase kinase; PI3K, phosphatidylinositol 3-kinase; PMN, polymorphonuclear leukocyte; TEB, terminal end bud; t10,c12-CLA, trans-10, cis-12 CLA.

Effect of CLA on established mammary tumors ErbB2 transgenic mice with mammary tumors 3–5 mm in the larger diameter. Mammary glands were prepared for whole mount analysis or fixed in formalin.

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in the longer diameter. Mammary tumors were frozen in liquid nitrogen immediately upon removal.

Preparation of mammary whole mounts, histology and immunohistochemistry
Mammary whole mounts from mammary glands 4 and 5 were prepared as described previously (12). Where indicated, the degree of development/branching of each whole mount was scored independently by each of the authors using a fractional scale between 1 and 4 (described in Figure 2), the values averaged and mean and standard error of the mean calculated from the averages.

Hematoxylin and eosin (H&E) staining, Ki67 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling immunohistochemistry and photography were as described previously (12).

Tumor lysate preparation and western blot analysis
Tumors were pulverized at liquid nitrogen temperature, then sonicated in a lysis buffer [1% Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 10 mM sodium phosphate, 10 mM sodium pyrophosphate, 5 mM sodium vanadate, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 2 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 20 μg/ml Pefabloc, 1 mM dithiothreitol, 1 μg/ml pepstatin A, 10 μg/ml aprotinin, and 10 mM sodium fluoride]. Lysates were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, then transferred to polyvinylidene difluoride membranes for western blot detection by antibodies to phospho-insulin-like growth factor I receptor (IGF-IR)-β (Tyr1135/1136/insulin receptor (Tyr1150/1151, #8908), IGF-IRβ (#3027), phopho-Her2/ErbB2 (Tyr877, #2241 and Tyr1221/1222, #2243), phospho-tyrosine (#408), phospho-MEK1/2 (Ser217/221, #9121), MEK1/2 (8922), phopho-p44/42 mitogen-activated protein kinase (Thr202/Tyr204, #9101), p44/42 mitogen-activated protein kinase (9102), phospho-P38 p85 (Tyr458/465/Tyr467, #9228) and P38 p85 (4292), phospho-Akt (Ser473, #4058) and Akt (#9272) from Cell Signaling (Danvers, MA) and Her2/ErbB2 (#2272) from Abcam (Cambridge, MA). Antibody to Hsc70 (SPA-820), obtained from Stressgen (Ann Arbor, MI), was used as a loading control.

Statistics
Log-rank analysis was used to statistically evaluate the Kaplan–Meier curves (percent mice tumor-free at each time and overall survival) with the Holm–Sidak method used to analyze multiple curves. Statistical analysis of survival time after the development of a palpable tumor, body weight, organ sizes, percent of Ki67 positive or terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling positive nuclei and the western blot data were tested by one-way analysis of variance, with the Holm–Sidak or Dunn’s method, as appropriate, used for pairwise multiple comparisons. Statistical differences between tumor diameters (Figure 1C) at each week were tested by t-test. A P value of <0.05 was considered statistically significant. Where shown, error bars are standard error of the mean.

Fig. 1. t10,c12-CLA accelerates mammary tumor development in ErbB2 transgenic mice irrespective of the time of its administration. (A, B) Tumorigenesis study. ErbB2 transgenic mice were fed control or 0.5% CLA-supplemented diets as follows. Group 1, mice were fed the control diet continuously throughout the experiment; group 2, mice were fed a 0.5% t10,c12-CLA-supplemented diet from 10 to 14 weeks of age, then returned to the control diet until the end of the experiment; group 3, mice were fed a 0.5% t10,c12-CLA diet continuously from 10 weeks of age; group 4, mice were fed the control diet until 21 weeks of age, the 0.5% t10,c12-CLA-supplemented diet from 21 to 25 weeks of age and then returned to the control diet for the duration of the experiment; group 5, mice were fed the control diet until 21 weeks of age, then switched to the 0.5% t10,c12-CLA-supplemented diet for the duration of the experiment; group 6, mice were fed a 0.5% t10,c12-CLA-supplemented diet continuously from 10 weeks of age (data from this latter group are reported in Table I). (A) and (B) are Kaplan–Meier plots showing percentage of tumor-free mice at various ages in mice fed control or 0.5% t10,c12-CLA-supplemented diets. In (A) mice were fed a t10,c12-CLA-supplemented diet continuously from 10 weeks of age until killing, or from 10 to 14 weeks of age, followed by a return to the control diet until killing. The control group was fed the control diet continuously. In (B) mice were fed a t10,c12-CLA-supplemented diet continuously from 21 weeks of age until killing, or from 21 to 25 weeks of age, followed by a return to the control diet until killing. The same control group as in (A) is shown in this panel. Latency was significantly decreased in mice fed the t10,c12-CLA diet (P < 0.001), but did not quite achieve statistical significance in the mice fed the t10,c12-CLA-supplemented diet continuously from 21 weeks of age (P = 0.068). The apparent accelerated tumor development in mice fed the t10,c12-CLA diet from 10 to 14 (A) or 21 to 25 (B) weeks of age was not statistically different than control. (C) Established tumor study. ErbB2 transgenic mice with palpable mammary tumors were fed control (9 mice, 12 tumors total) or 0.5% t10,c12-CLA-supplemented diets (11 mice, 12 tumors total) for 4 weeks, and tumor diameter measured weekly. Each point represents the mean ± standard error of the mean of the diameter of the 12 tumors per group that were present at time zero. *Statistically different from control (P = 0.008 and 0.018 at 3 and 4 weeks, respectively). Inset: the percentage of mice in each group developing a new tumor during treatment (1 of 9 mice in the control group; 9 of 11 mice in the CLA group).
Results

t10,c12-CLA stimulates mammary tumorigenesis independently of the time of its administration

Previously, we found that continuous feeding of a diet supplemented with 0.5% t10,c12-CLA from weaning, or from 10 weeks of age, markedly accelerated mammary tumor development and lung metastasis in ErbB2 transgenic mice (12). In the current study, we asked whether short-term administration of this CLA isomer might be deleterious. We focused on two 4-week time periods: (i) post-puberty, from 10 to 14 weeks of age, and (ii) immediately prior to the time when palpable mammary tumors were expected, from 21 to 25 weeks of age. At the former time, although all the cells can be considered ‘initiated’ since ErbB2 is overexpressed, preneoplastic lesions are not readily detected in the mammary gland. In contrast, although palpable tumors are not seen at 21 weeks of age, preneoplastic and subclinical neoplastic lesions are expected to be present. For comparison purposes, two additional groups of mice were fed the t10,c12-CLA-supplemented diet continuously from 10 or 21 weeks of age. The latter group also allowed us to examine the effect of delayed t10,c12-CLA feeding on tumorigenesis. Finally, an additional control group was fed a diet supplemented with 0.5% c9,11-CLA from 10 weeks of age.

Consistent with our previous studies, continuous supplementation with t10,c12-CLA from 10 weeks of age markedly stimulated mammary tumorigenesis in ErbB2 transgenic mice, with latency, the time when 50% of the mice in each group had a palpable tumor, decreased from 40.6 weeks in the control to 26.6 weeks in the CLA group (Figure 1A and Table I, P < 0.001). Overall survival time, calculated based on the time each mouse had to be killed because of tumor size, was also significantly decreased in the CLA group (Table I). In contrast to the effects with t10,c12-CLA, c9,11-CLA did not have any significant effect on latency or survival (Table I).

Short-term administration of t10,c12-CLA appeared to temporarily accelerate mammary tumor development. To quantify this, we compared weekly differences in the percent of tumor-bearing mice between the control and short-term supplementation groups beginning ~10 weeks (determined empirically) after the end of the supplementation. The group fed the t10,c12-CLA diet between 10 and 14 weeks of age had an average of 14.3% (±1.6) more tumor-bearing animals each week in the 15-week time period between 25 and 40 weeks of age (Figure 1A). Similarly, in the group fed t10,c12-CLA between 21 and 25 weeks of age, there were 14.2% (±1.4) more tumor-bearing animals each week in the 10-week time period between 36 and 46 weeks of age (Figure 1B). However, for each of the short-term supplementation groups, overall survival and survival time after detection of a palpable tumor did not differ from control (Table I).

Initiation of continuous t10,c12-CLA supplementation starting just prior to expected tumor development (21 weeks of age) accelerated mammary tumor development compared with control (Figure 1B and Table I), although the decreased latency just failed to achieve statistical significance (P = 0.068), probably because the progression timeline was so far advanced. Importantly, CLA was considerably more deleterious when started at this later age, since both overall survival of the mice as well as time of survival after detection of a palpable tumor were significantly shortened compared with the control group (Table I, P < 0.01). The latter measurement suggests that tumor growth rate was considerably enhanced by this t10,c12-CLA supplementation protocol.

t10,c12-CLA stimulates the growth of established ErbB2-overexpressing mammary tumors

The stimulation of tumorigenesis in the older mice, as well as their decreased survival time, suggested that in addition to enhancing growth of preneoplastic/subclinical neoplastic lesions, t10,c12-CLA supplementation might also stimulate the growth of established mammary tumors. Since women with breast cancer might take a CLA supplement, we addressed this question by randomizing tumor-bearing mice into two groups and feeding them control or 0.5% t10,c12-CLA-supplemented diets for 4 weeks. As seen in Figure 1C, growth of established tumors was significantly increased at the 3- and 4-week time points. Even more significantly, however, during the treatment period, 9 out of 11 mice in the t10,c12-CLA group developed a new palpable tumor. By comparison, only one out of nine mice in the control group developed a new tumor (Figure 1C, inset).

Long-term but not short-term administration of t10,c12-CLA increases the size of the kidney, liver, heart and spleen

We reported previously that when ErbB2 transgenic mice were fed the t10,c12-CLA-supplemented diet from weaning, the weights of the liver, heart and spleen were significantly increased compared with mice fed the control or c9,11-CLA-supplemented diets (12). In the current study, we asked whether delaying the start of the t10,c12-CLA supplementation to 10 or 21 weeks of age, or short-term supplementation for only 4 weeks, would have an adverse effect on organ size. As seen in supplementary Table I (available at Carcinogenesis Online), continuous feeding of the t10,c12-CLA diet from 10 or 21 weeks of age not only resulted in a doubling of the relative size (organ weight per 100 g body wt) of the heart, liver and spleen but also increased the relative kidney size by 40%. These changes were not seen in mice fed the t10,c12-CLA-supplemented diet for only 4 weeks. No changes in organ sizes were observed in the mice fed the diet supplemented with 0.5% c9,11-CLA (supplementary Table I is available at Carcinogenesis Online).

Short-term administration of t10,c12-CLA to ErbB2 transgenic mice induces morphological changes in the mammary gland

Long-term supplementation with 0.5% t10,c12-CLA but not with c9,11-CLA induced a dramatic lobular hyperplasia of the mammary epithelium, which was associated with the increased mammary tumorigenesis in this group (12). Since a short, 4 week, supplementation with t10,c12-CLA resulted in a temporary acceleration of mammary tumor development in the ErbB2 transgenic mice, we asked whether 4 weeks were sufficient to induce morphological changes in the mammary gland and, if so, whether these changes were maintained once the mice were switched back to the control diet. Figure 2A and B shows that the mammary glands of mice fed the diet containing 0.5% t10,c12-CLA for 4 weeks were more developed, with significantly

Table I. Effect of time of administration of CLA on mammary tumorigenesis and survival

<table>
<thead>
<tr>
<th>Time Points</th>
<th>Control</th>
<th>10,12-CLA, weeks 10–14</th>
<th>10,12-CLA, weeks 10 to end</th>
<th>10,12-CLA, weeks 21–25</th>
<th>10,12-CLA, weeks 21 to end</th>
<th>9,11-CLA, weeks 10 to end</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor latency (median age in weeks)</td>
<td>40.6*</td>
<td>35.6*</td>
<td>26.6*</td>
<td>37.6*</td>
<td>34.6*</td>
<td>36.6*</td>
</tr>
<tr>
<td>Time of first tumor (age in weeks)</td>
<td>38.9 ± 1.7a</td>
<td>36.4 ± 2.4a</td>
<td>27.3 ± 1.0b</td>
<td>38.1 ± 1.7a</td>
<td>34.6 ± 1.3a</td>
<td>38.9 ± 1.9a</td>
</tr>
<tr>
<td>Overall survival (age in weeks)</td>
<td>46.4 ± 2.0*</td>
<td>44.9 ± 2.4*</td>
<td>33.6 ± 1.0*</td>
<td>45.6 ± 2.0*</td>
<td>39.0 ± 1.3*</td>
<td>46.6 ± 2.1*</td>
</tr>
<tr>
<td>Survival time after detection of palpable tumor (weeks)</td>
<td>7.49 ± 0.63a</td>
<td>6.84 ± 0.77a</td>
<td>6.23 ± 0.44a</td>
<td>6.43 ± 0.66a</td>
<td>4.57 ± 0.27b</td>
<td>6.60 ± 0.54a</td>
</tr>
</tbody>
</table>

Latency: age at which 50% of the mice had developed a tumor. Overall survival: age at which mouse had to be killed because of tumor size. Numbers without a common letter are statistically different.
increased branching, ductal budding and lobular development when compared with mice fed the control diet or the diet supplemented with 0.5% c9,t11-CLA. Concurrently, the length of the epithelium from the lymph node to the edge of the fat pad was significantly decreased (data not shown). These t10,c12-CLA-induced morphological changes were independent of estrous cycle stage at the time of killing. From the H&E sections, it can be seen that in the mice fed t10,c12-CLA, there was significant loss of the white adipose tissue in the mammary gland, and almost complete loss of the brown adipose tissue (Figure 2C). Additionally, there was a marked infiltration of polymorphonuclear leukocytes (PMNs) into the stroma surrounding the mammary epithelium (Figure 2C, vi, arrow), and mammary lymph node size was increased by 35% (Figure 2A, right panel). These differences were not seen in mice fed c9,t11-CLA.

Since one or more of these changes could be associated with the accelerated tumor development, we asked whether they persisted in mice given the t10,c12-CLA-supplemented diet for 4 weeks and then switched back to the control diet for the duration of the tumorigenesis study. As seen in Figure 2D, although prolonged continuous t10,c12-CLA administration stimulated an extensive and statistically significant lobular and ductal hyperplasia, mammary development scores in the mice fed the t10,c12-CLA diet for only 4 weeks, before being returned to the control diet, were not significantly different from those in the mice fed the control diet. This would suggest that the...
morphological changes were reversible. In contrast, mammary lymph node size, which was significantly increased by long-term administration of t10,c12-CLA, remained elevated in mice fed this isomer for 4 weeks before being returned to the control diet (Figure 2D). No effect of c9,t11-CLA was noted on lymph node size.

Mammary glands in the groups fed t10,c12-CLA continuously from 10 or 21 weeks of age were very small compared with the control and c9,t11-CLA groups, and had lost all of the brown adipose tissue and the majority of the white adipose tissue (data not shown). PMN infiltration into the ductal stroma was marked (data not shown). When the t10,c12-CLA diet was fed for only 4 weeks before returning the mice to the control diet, the mammary glands were noticeably smaller than those in the control group, although larger than those fed t10,c12-CLA continuously. Return to the control diet resulted in repopulation with white adipose tissue, although very little brown adipose tissue; PMN infiltration in the mammary ductal stroma of these mice was variable.

CLA alters proliferation and apoptosis of ErbB2-overexpressing mammary epithelium in a time-dependent manner

We recently reported that a short, 10 day, dietary supplementation with t10,c12-CLA starting at 10 weeks of age significantly stimulated proliferation of ErbB2-overexpressing mammary epithelium, suggesting that this may be a major factor contributing to the accelerated mammary tumor development (12). In the current experiments, we asked whether this stimulatory effect on the mammary epithelium was maintained with prolonged feeding of the t10,c12-CLA isomer. Two independent studies with ErbB2 transgenic mice were performed. In the first, mice were fed control or 0.5% CLA-supplemented diets from 10 to 14 weeks of age and then killed. In the second, supplementation was initiated at 10 weeks of age, and continued for the lifetime of the mice (defined as the time when a mouse had to be killed because of tumor size). Proliferation was evaluated by Ki67 immunohistochemistry. As seen in Figure 3 (left panel), the Ki67 labeling index was modestly increased in the ductal and lobular epithelium of mice fed the t10,c12-CLA-supplemented diet for 4 weeks; however, this was not statistically significant, unlike the situation with 10 days of feeding [Figure 3, (12)]. The third epithelial structure examined was the terminal end bud (TEB). In young animals, this is the bulbous structure at the end of the growing ducts that consists of multiple cell layers. In the older, transgenic ErbB2 mice in these studies, however, preneoplastic and small neoplastic lesions also have multiple cell layers when examined by cross-section, so the structures referred to as TEBs/lesions in the two older groups are most probably lesions, rather than TEBs that have generally disappeared by this age. In the lesions of mice fed the control diet for 4 weeks, proliferation was quite high and was not further increased by CLA. In contrast, there was a marked increase in the labeling index in TEBs of mice fed the diet for only 10 days.

In contrast to the mice fed CLA diets for 10 days or 4 weeks, the Ki67 labeling index in all epithelial structures was slightly but significantly decreased by lifetime supplementation of the diet with t10,c12-CLA. An even greater decrease was observed in the ducts and lesions of mice supplemented with c9,t11-CLA (Figure 3). Although we cannot perform a statistical analysis because the experiments were performed independently, it is noteworthy that the proliferative index in the control mice markedly increased with age. This is not unexpected given the overexpression of ErbB2 in the epithelium, and it could explain the apparent altered CLA responsiveness in the older animals.

Apoptosis in the mammary epithelium was examined concurrently by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling immunochemistry. Overall, the apoptotic index (Figure 3, right panel) was much lower than the proliferative index. However, t10,c12-CLA stimulated apoptosis in the mammary epithelium of mice fed this isomer for 10 days or 4 weeks. Interestingly, this effect was lost after long-term administration (‘lifetime’), when a significant decrease in apoptosis was observed in each of the mammary epithelial structures from the t10,c12-CLA group. In comparison, c9,t11-CLA stimulated apoptosis in the mammary epithelium after 4 weeks supplementation, and had no effect or modestly decreased apoptosis when supplemented for the lifetime of the mice.

t10,c12-CLA stimulates survival and proliferative signaling

To gain leads as to how t10,c12-CLA might be affecting tumorigenesis, two major signaling pathways were examined in mammary tumors from mice fed control or CLA-supplemented diets. As seen in Figure 4, phosphorylation of PI3K and Akt was increased 12.0- and 9.7-fold, respectively, in tumors from mice fed the t10,c12-CLA-supplemented diet. Although total PI3K and Akt each decreased by 50%, the increased phosphorylation of these two proteins far exceeded the decrease in total protein, suggesting activation of survival signaling in these tumors. Additionally, phosphorylation of MEK and extracellular signal-regulated kinase (ERK) was increased 3.2- and 3.7-fold in the tumors from mice fed the t10,c12-CLA-supplemented diet, concurrent with a 56% decrease in total MEK and a 1.1-fold increase in total ERK, suggesting an increase in proliferative pathways. No significant differences were observed in tumors from mice fed c9,t11-CLA. We tested the hypothesis that this stimulatory effect was mediated by an increased signaling through ErbB2. Since ErbB2 is activated by phosphorylation, western blot analysis was used to examine the phosphorylation status of ErbB2. No significant difference in phospho-Tyr877 (Figure 5) or phospho-Tyr1221/1222 (data not shown) was observed among the three groups. Moreover, total ErbB2 was not changed. We also used an antibody against total phospho-tyrosine, but did not find any significant change at ~185 kDa, the molecular weight of ErbB2 (Figure 5). We next asked whether t10,c12-CLA activated the IGF-IR, since both the Akt and ERK pathways are downstream targets of this receptor (23,24). Indeed this was the case, and Figure 5 shows that phosphorylation of IGF-IR and/or insulin receptor (the phospho-antibody detects both) was significantly increased 7-fold in mice fed t10,c12-CLA. Total IGF-IR was not significantly changed by dietary CLA.

Discussion

The present study confirms our earlier report that supplementation of the diet of ErbB2 transgenic mice with t10,c12-CLA stimulates mammary tumorigenesis from initiated mammary epithelial cells, and significantly extends the previous work to demonstrate that this CLA isomer also stimulates the growth of established mammary tumors and accelerates tumor development from preneoplastic and subclinical neoplastic lesions. This increase was associated with an imbalance in the proliferation and apoptosis programs of the mammary epithelium, altered mammary gland morphology and increased signaling through both proliferation and survival pathways in mammary tumors, in association with activation of the IGF-I/insulin receptor.

Clinical implications

Approximately 25–30% of human breast cancers overexpress Her2/ErbB2 (15,16). Our experiments strongly suggest that t10,c12-CLA, widely available in CLA supplements, is deleterious to this group of women. A short 4 week supplementation with t10,c12-CLA not only stimulated the growth of established mammary tumors, but also dramatically increased the appearance of new tumors. This latter observation implies that the growth of preexisting subclinical neoplasias, as well as palpable cancers, was markedly accelerated during this 4 week period, and together with the marked change in the mammary stroma, supports the biological significance of the accelerated tumor development in mice receiving the 4 week supplement at the earlier stages of the progression timeline (Figure 1). Although it appeared as if this acceleration was reversible after the mice were returned to the control diet, translation of this observation to the human population might suggest that preexisting lesions within the breast might get a jump start, and depending on the length of supplementation might progress to clinical breast cancer, requiring treatment sooner than would have
occurred in the absence of supplementation. Related to this observation, it is particularly unsettling to note the marked decrease in survival time after detection of a palpable tumor, when long-term supplementation of t10,c12-CLA was initiated just prior to the expected time of detection of mammary tumors. Specifically, the decreased survival to only 4.6 weeks in mice supplemented starting at 21 weeks of age, compared with the control survival time of 7.5 weeks, suggests that t10,c12-CLA dramatically increased the tumor growth rate.

As an initial approach to understanding the mechanism by which t10,c12-CLA exerted its effects, we examined the effect of each CLA isomer on the proliferative and apoptotic indices in the mammary epithelium of ErbB2-overexpressing mice. Three separate experiments were performed in which 10-week-old mice were fed control or CLA-supplemented diets for 10 days (n = 5 per group), 4 weeks (n = 7 per group) or until killing because of tumor size (‘lifetime’) (n = 7 per group), starting at 70 days of age. These three experiments were performed independently. The data for the 10 days feeding have been reported previously (12) and are presented here for comparison purposes. Left panels: Ki67 immunohistochemistry. For the control, c9,t11-CLA and t10,c12-CLA groups, respectively, the number of mammary structures counted were as follows. Ten days: ducts 62, 60, 66; lobules 50, 53, 57 and TEBs 60, 58, 57. Four weeks: ducts 72, 84, 84; lobules 66, 77, 84 and TEBs/lesions 45, 77, 79. Lifetime: ducts 84, 87, 70; lobules 83, 79, 82 and TEBs/lesions 86, 79, 56. Right panels: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) immunohistochemistry. For the control, c9,t11-CLA and t10,c12-CLA groups, respectively, the number of mammary structures counted were as follows. Ten days: ducts 66, 71, 72; lobules 52, 43, 59 and TEBs/lesions 86, 79, 56. Four weeks: ducts 77, 86, 85; lobules 72, 79, 84 and TEBs/lesions 59, 75, 81. Lifetime: ducts 87, 84, 70; lobules 85, 84, 78 and TEBs/lesions 91, 87, 79. Within a specific time period, bars without a common letter are statistically different.

Fig. 3. Effect of CLA feeding for different times on proliferation and apoptosis of mammary epithelial cells. ErbB2 transgenic mice were fed control or 0.5% CLA-supplemented diets for 10 days (n = 5 per group), 4 weeks (n = 7 per group) or until killing because of tumor size (‘lifetime’) (n = 7 per group), starting at 70 days of age. These three experiments were performed independently. The data for the 10 days feeding have been reported previously (12) and are presented here for comparison purposes. Left panels: Ki67 immunohistochemistry. For the control, c9,t11-CLA and t10,c12-CLA groups, respectively, the number of mammary structures counted were as follows. Ten days: ducts 62, 60, 66; lobules 50, 53, 57 and TEBs 60, 58, 57. Four weeks: ducts 72, 84, 84; lobules 66, 77, 84 and TEBs/lesions 45, 77, 79. Lifetime: ducts 84, 87, 70; lobules 83, 79, 82 and TEBs/lesions 86, 79, 56. Right panels: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) immunohistochemistry. For the control, c9,t11-CLA and t10,c12-CLA groups, respectively, the number of mammary structures counted were as follows. Ten days: ducts 66, 71, 72; lobules 52, 43, 59 and TEBs/lesions 86, 79, 56. Four weeks: ducts 77, 86, 85; lobules 72, 79, 84 and TEBs/lesions 59, 75, 81. Lifetime: ducts 87, 84, 70; lobules 85, 84, 78 and TEBs/lesions 91, 87, 79. Within a specific time period, bars without a common letter are statistically different.

t10,c12-CLA alters proliferative and apoptotic indices within the epithelium in a time- or age-dependent manner

As an initial approach to understanding the mechanism by which t10,c12-CLA exerted its effects, we examined the effect of each CLA isomer on the proliferative and apoptotic indices in the mammary epithelium of ErbB2-overexpressing mice. Three separate experiments were performed in which 10-week-old mice were fed control or CLA-supplemented diets for 10 days, 4 weeks or for their lifetime. As we reported previously (12), both proliferation and apoptosis were significantly increased in the mammary epithelium of mice fed the t10,c12-CLA-supplemented diet for 10 days, although proliferation predominated. With longer administration (4 weeks or lifetime), and concomitantly as the mice became older, the stimulatory effect on proliferation was no longer observed. This could suggest that the epithelium of the older mice that consumed the diet for the longest time had become resistant to the stimulatory effect of t10,c12-CLA. Alternatively, and more likely given the increased proliferative rate in the controls, proliferation had already reached a threshold beyond which a further increase could not occur. In contrast, the apoptotic rate was elevated in both CLA groups after 4 weeks of feeding. This observation is consistent with in vitro data demonstrating that t10,c12-CLA stimulates apoptosis (1,7,25), and in the current, in vivo experiment may suggest that the potential detrimental effects of 4 weeks feeding on tumorigenesis are truly reversible. Finally, apoptosis was significantly decreased in the mammary
epithelium of mice fed the t10,c12-CLA-supplemented diet for their lifetime, which might contribute to the ability of this isomer to stimulate tumor growth. Whether the dynamic changes in the proliferation and apoptosis indices relate to the length of the feeding, to the age of the mouse or to the degree of preneoplasia/neoplasia cannot be assessed from these data.

It was not possible to conduct more detailed mechanistic studies within the epithelium. First, because of the marked change in the stromal component of the mammary gland in the t10,c12-CLA-supplemented mice (loss of adipocytes, increased PMN), whole mammary gland comparisons would not be valid. Furthermore, digestion of the epithelial cells from the glands for comparative purposes disrupts the normal signaling pathways and alters phosphorylation status. Finally, although we did attempt immunohistochemical comparisons using phospho-specific antibodies for ERK and Akt, we were not able to achieve specific quantifiable staining.

t10,c12-CLA stimulates mammary tumor growth by activating proliferative and survival signaling

Since we were not able to pursue detailed studies on the epithelium, we utilized mammary tumors to investigate potential mechanisms by which t10,c12-CLA might stimulate growth. Our data demonstrated that t10,c12-CLA stimulated mammary tumor growth by increasing both survival and proliferation, as evidenced by activation of both the PI3K/Akt and MEK/ERK pathways. Akt is a serine–threonine protein kinase that is activated in response to increased PI3K signaling, and regulates numerous cellular functions including proliferation and survival in breast cancer cells (26). ERK1 and ERK2, which are activated by MEK phosphorylation of threonine and tyrosine residues, are kinases that phosphorylate a large number of substrates (27). Sustained ERK activation, as may occur in breast cancer (28), leads to an increase in proliferation (29). The increased activation of ERK seen in our studies is consistent with that reported by Brown et al. (30) in human stromal vascular cells treated with t10,c12-CLA, but opposite to that found in tumor cells in vitro, where a mixture of CLA isomers was shown to inhibit phosphorylation of ERK (31) and Akt (32) in association with an inhibition of cell growth. This latter difference may reflect the tumor environment in vivo, where the markedly altered stroma as well as alterations in systemic hormonal or immune functions may supply positive and sustained signals for tumor growth.

Our initial expectation was that the stimulatory effect of t10,c12-CLA would involve increased signaling through ErbB2, since

Fig. 4. t10,c12-CLA activates the PI3K/Akt and MEK/ERK pathways. ErbB2 transgenic mice were fed control or 0.5% CLA-supplemented diets continuously from 24 or 70 days of age, and protein expression evaluated in mammary tumors. (A) Phospho-PI3K p85 (Tyr458)/p55 (Tyr199), total PI3K, phospho-Akt (Ser473), total Akt, phospho-MEK (Ser217/221), total MEK, phospho-ERK1/2 (Thr202/Tyr204) and total ERK1/2 were measured by western blot in eight mammary tumors per group. Hsc70 was used as a loading control. (B) The western blots were quantified by densitometry. Mean ± standard error of the mean of the ratio of phospho-PI3K p85 to PI3K p85, phospho-Akt (Ser473) to Akt, phospho-MEK to MEK and phospho-ERK1/2 to ERK1/2. Bars without a common letter are statistically different.

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overexpression of this receptor is responsible for tumor development in this model. This did not appear to be the case, however, since phosphorylation of the two ErbB2 sites we were able to measure as well as tyrosine phosphorylation of a protein at the expected size of ErbB2 was not altered. Together with our previous data that short-term supplementation with t10,c12-CLA stimulated mammary epithelial proliferation in FVB wild-type mice (12), this suggests that activation or overexpression of ErbB2 is not required for the stimulatory effect of t10,c12-CLA. IGF-IR, a transmembrane tyrosine kinase receptor, which is activated by binding of its ligands IGF-I and IGF-II and which plays an important role in mediating breast cancer growth, survival and metastasis (23,33), was an attractive alternative candidate, since it shares the same Akt/ERK downstream pathways. Our observation that its phosphorylation was stimulated by t10,c12-CLA supports its potential role in transducing the survival/proliferation signal. This CLA isomer has been shown previously to induce hyperinsulinemia in mice (34), and since insulin can activate the IGF-IR as well as the insulin receptor (35), this may explain the increased signaling through the IGF-I/insulin receptor pathway that we observed in this study. Future studies will address the role of this pathway in mediating the effect of CLA in the ErbB2 transgenic mouse model.

In summary, our data demonstrate that t10,c12-CLA accelerates mammary tumorigenesis and stimulates the growth of established tumors and preneoplastic lesions in a model of ErbB2-overexpressing mammary cancer, and that stimulation is associated with an increased activation of IGF-I/insulin receptor and its downstream targets Akt and ERK. Notably, these effects were isomer specific, and were not seen in mice supplemented with c9,t11-CLA. These data strongly suggest that a readily achievable dose of a CLA supplement containing the t10,c12 isomer (12) may be deleterious to women whose breast epithelium or tumor overexpresses Her2/ErbB2, as well, potentially, to other women at risk for breast cancer.

Supplementary material

Supplementary Table I can be found at http://carcin.oxfordjournals.org/

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References


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