Exposure to Flaxseed or Purified Lignan During Lactation Influences Rat Mammary Gland Structures

Wendy E. Ward, Fanny O. Jiang, and Lilian U. Thompson

Abstract: Previous investigation demonstrated that feeding a 10% flaxseed (10F) diet during pregnancy and lactation enhanced the differentiation of highly proliferative terminal end bud (TEB) structures of rat mammary gland into less proliferative alveolar buds and lobules. From this study, it was hypothesized that the lignan component in flaxseed mediated the observed effects. Because mammary glands with more TEBs are more susceptible to carcinogens, exposure to flaxseed during early postnatal life may reduce the risk of developing mammary cancer. Our objectives were to elucidate whether exposure to flaxseed during lactation only and during pregnancy and lactation can similarly influence the differentiation of mammary gland structures and also to identify whether the lignan component of flaxseed is the biologically active agent. Offspring were exposed to a 10F diet or a dose of purified lignan equivalent to that in a 10F diet (10S) during lactation only or from lactation to postnatal Day 50. Compared with controls, exposure to 10F or 10S during lactation only or from lactation to postnatal Day 50 reduced the number of TEBs and resulted in a rise in the number of alveolar buds. In conclusion, exposure to flaxseed or its purified lignan during lactation is a critical period in which mammary gland development may be promoted by enhancing the differentiation of mammary gland structures. However, continuous exposure, particularly to purified lignans, resulted in the most differentiation of the mammary gland. The next step is to determine whether the changes in mammary gland structures are chemopreventive in rats challenged with a carcinogen.

Introduction

Flaxseed is the richest source of secoisolariciresinol diglycoside (SDG), the mammalian lignan precursor from which the two major lignans, enterolactone and enterodiol, are formed by the action of colonic bacteria (1). Enterolactone and enterodiol share a structure similar to 17β-estradiol and have been shown to have weak estrogenic or antiestrogenic activity in in vitro and in vivo studies, depending on the dose, duration of administration, and stage of development (2–5).

It has been shown that mammary glands in animals with a higher proportion of differentiated structures are less susceptible to carcinogens than those in animals with less differentiated structures (6,7). Because sex steroid hormones such as estrogen and progesterone are proven mediators of mammary gland development and differentiation (8), the potential biological action of natural dietary estrogens on mammary gland development has been studied (9–11) as a possible stimulus of mammary gland development and differentiation.

Rats exposed to genistein, another phytoestrogen with structural similarities to estrogen and possibly estrogenic or antiestrogenic activity, during perinatal (11), neonatal (10), or prepubertal (12) life experienced a delay in the development of mammary tumors and also had fewer tumors at 190 days than rats that had not been exposed to genistein. The chemopreventive effect was attributed to an overall reduction in the number of terminal end buds (TEBs) by promoting TEB differentiation and, more specifically, a suppression of cell proliferation in TEB structures as the number of cells in the S phase of the cell cycle was significantly reduced with genistein exposure (10–12).

Previous investigation in our laboratory demonstrated that feeding flaxseed or purified SDG during pregnancy and lactation altered mammary gland development (9). Feeding a 10% flaxseed (10F) diet during pregnancy and lactation enhanced the differentiation of mammary gland structures, resulting in fewer TEBs and more alveolar buds (ABs) (9). No study has determined whether these effects were mediated by exposing rats during pregnancy, lactation, or both stages of development. Also, it was uncertain whether the lignan component of flaxseed (i.e., SDG) mediated the observed effects on mammary gland structures. Thus the overall objective of this study was to determine whether lactation only is a critical stage of flaxseed feeding that impacts on mammary gland maturation and differentiation and whether the lignan component of flaxseed was responsible for the effects observed. Also, at the end of lactation, rats were switched to a basal diet (BD) or remained on their mother’s diet to evaluate whether continuous exposure to purified SDG or flaxseed resulted in a greater proportion of differentiated mammary gland structures than exposure only during lactation. It was previously established that lignans are...
transferred to the offspring via rat dam’s milk, as indicated by the recovery of radioactivity in the offspring of lactating dams administered [3H]SDG (13).

Materials and Methods

Diets

The composition of the BD was based on the semi-purified AIN-93G diet (14). The BD was supplemented with 10F (Linnott variety, Omega Products, Melfort, SK, Canada) after correction for protein, fat, and fiber contributed by flaxseed, as previously described (13), or purified SDG (10S) equivalent to the level of SDG present in a diet containing 10% flaxseed (10S). Because it was determined by high-performance liquid chromatography analysis that 1.77 mg of SDG was present in 1 g of flaxseed (15), the 10S diet contained 17.7 mg/100 g diet. All dietary ingredients were from Dyets (Bethlehem, PA) and were stored at 4°C. Fresh water and their respective diets throughout the study. Offspring/litter from each dam were killed by carbon dioxide inhalation followed by a cervical dislocation. As previously described by Tou and Thompson (9), the pelt was removed, stretched, and pinned on a corkboard and then fixed in 10% phosphate-buffered formalin for 48 hours. The right abdominal gland (Gland 4) was then dissected from the pelt and processed for the whole mount. Mammary glands were defatted in acetone for 2–10 days depending on the degree of fat present on the gland. Glands were hydrated in a series of decreasing concentrations of ethanol (100%, 95%, and 70%) for one hour each and then stored in distilled water overnight. Glands were stained in 0.015% toluidine blue for two hours. After a wash with distilled water, glands were destained in 100% methanol and 70% ethanol for 30 minutes each. Destained glands were fixed in 4% ammonium molybdate for 30 minutes and then stored overnight in distilled water. Glands were then rehydrated in increasing concentrations of ethanol (70%, 95%, and 100%) for one hour at each concentration and stored in xylene overnight. Mammary glands were stored in heat-sealed pouches (Kapak, Minneapolis, MN) with enough methyl salicylate to ensure that the gland did not dry out.

Counting of Mammary Gland Structures

Mammary gland structures including TEBs, ABs, and lobules were counted, as previously described (9), in a blinded manner, such that the investigator was unaware of the diet an animal had received. Ten 1-mm² areas were randomly selected in the distal portion of the mammary gland, and the number of TEBs, ABs, and lobule structures was determined by examination under a stereomicroscope at ×3.0 magnification.

Statistical Analyses

Statistical analyses were performed using SigmaStat software (version 2.0, Jandel Scientific, San Rafael, CA). For data that were normally distributed (TEB density, AB density, and body weight at PND 2, PND 21, and PND 50), a one-way analysis of variance (ANOVA) followed by Tukey’s test was used to determine differences among treatment groups. For data that did not follow a normal distribution (lobule density, food intake, and SDG intake), a Kruskal-Wallis one-way ANOVA on ranks followed by Dunn’s test was used to detect differences among groups. Two-way ANOVA was performed to determine whether differences in any outcome were due to treatment or timing of flaxseed or SDG exposure. Differences were considered significant if \( p \leq 0.05 \). Values are means ± SEM, except in Table 2.

Results

Total Food and SDG Intake From the End of Lactation (PND 21) Through PND 50 and Weight Growth

From the end of lactation (PND 21) through PND 50, the total food intakes of offspring did not differ among any treatment groups. During this same time period, lignan (SDG) in-
take was significantly higher among rats exposed to the 10F or 10S diet than in those fed BD. The SDG intake in the 10F and 10S diet groups was similar. Body weights did not differ among groups at PND 2, at the end of lactation (PND 21), or at necropsy (PND 50) (Table 1).

Mammary Gland Structures

Exposure to the 10F or 10S diet, during lactation only or continuously, reduced (p < 0.05) the density of TEBs compared with rats exposed to BD (Figure 1). The reduction in the density of TEBs was attributed to the enhanced differentiation of TEBs to ABs, inasmuch as the density of ABs among rats exposed to 10S during lactation only or to 10F or 10S during lactation through PND 50 was higher (p < 0.05) than in the BD group (Figure 1). The density of ABs among rats exposed to 10F during lactation only was higher, but not significantly higher, than in the BD group. Lobule density was only affected by continuous exposure to the 10S diet. The density of lobules among rats continuously exposed to 10S was higher (p < 0.05) than among rats exposed to 10S during lactation only or BD (Figure 1), but this difference was small.

Two-way ANOVA indicated that 10F and 10S treatment affected TEB (p < 0.05) and AB (p < 0.05) density, but only 10S affected lobule density (p < 0.05). The timing of exposure affected AB (p < 0.05) and lobule (p < 0.05), but not TEB, density. There were no significant interactions between the treatments (BD, 10S, or 10F) and the timing of exposure (lactation only or continuous exposure).

Because one of the objectives of this study was to determine the critical stage of flaxseed or lignan exposure on mammary gland development, particularly the differentiation of proliferative TEBs, we directly compared the changes in the density of TEB structures that occurred in this study with those reported in our previous study (9), in which rats were exposed to a 10F diet during pregnancy and lactation or from pregnancy through PND 50 (Table 2). The density of TEBs of the treatment groups was expressed as a function of the mean value of the respective control groups.

### Table 1. Total Food and SDG Intake From the End of Lactation Through PND 50 and Weight Growth at PND 2, 21, and 50

<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>Food Intake, b g/day</th>
<th>SDG Intake, b µmol/day</th>
<th>Weight, g PND 2</th>
<th>Weight, g PND 21 c</th>
<th>Weight, g PND 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD-BD</td>
<td>14.48 ± 0.15</td>
<td>0</td>
<td>6.55 ± 0.29</td>
<td>57.00 ± 1.73</td>
<td>216.22 ± 5.65</td>
</tr>
<tr>
<td>10S-BD</td>
<td>14.65 ± 0.23</td>
<td>0</td>
<td>5.86 ± 0.14</td>
<td>54.27 ± 0.82</td>
<td>199.55 ± 5.10</td>
</tr>
<tr>
<td>10S-10S</td>
<td>14.62 ± 0.28</td>
<td>4.28 ± 2.41</td>
<td>6.06 ± 0.14</td>
<td>53.45 ± 0.94</td>
<td>198.00 ± 4.97</td>
</tr>
<tr>
<td>10F-BD</td>
<td>13.82 ± 0.38</td>
<td>0</td>
<td>6.53 ± 0.26</td>
<td>54.86 ± 1.67</td>
<td>214.43 ± 7.52</td>
</tr>
<tr>
<td>10F-10F</td>
<td>14.75 ± 0.84</td>
<td>4.32 ± 0.06</td>
<td>6.41 ± 0.28</td>
<td>56.67 ± 1.54</td>
<td>219.89 ± 6.38</td>
</tr>
</tbody>
</table>

a: Values are means ± SEM; n = 7–8/group. BD-BD, continuous basal diet; 10F-BD and 10S-BD, exposure to 10% flaxseed diet or purified secoisolariciresinol (SDG) at a level equivalent to that in a 10% flaxseed diet, respectively, during lactation only; 10F-10F and 10S-10S, continuous exposure to 10% flaxseed diet or purified SDG at a level equivalent to that in the 10% flaxseed diet.


c: Weight was measured at the end of lactation, immediately before weaning.

Figure 1. Effect of lactation or continuous exposure to 10% flaxseed (10F) or equivalent quantity of lignins in a 10% flaxseed diet (10S) on density of mammary gland terminal end buds (TEBs), alveolar buds (ABs), and lobules. Values are means ± SEM; n = 7–8/group. BD-BD, control group exposed to basal diet (BD) throughout study; 10F-BD and 10S-BD, group exposed to diet supplemented with 10F or 10S, respectively, during lactation and then BD after lactation through postnatal Day (PND) 50. 10F-10F or 10S-10S group was continuously exposed to 10F or 10S diet, respectively, during lactation through PND 50. Values with different letters (a, b) are significantly different.
Table 2. Effect of the Timing of Exposure to 10F or 10S Diet on Percent Change in Density of TEB Structures

<table>
<thead>
<tr>
<th>Diet Condition</th>
<th>10F Diet</th>
<th>10S Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy and lactation only</td>
<td>66</td>
<td>75</td>
</tr>
<tr>
<td>Lactation only</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Pregnancy through PND 50</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Lactation through PND 50</td>
<td>78</td>
<td>63</td>
</tr>
</tbody>
</table>

*a:* Values are calculated as $100 \times \text{mean of density of terminal end buds (TEB) of treatment group} / \text{mean of respective control group that received BD}; n = 6–8 rats/group.

*b:* Based on findings reported by Tou and Thompson (9). Rats were not exposed to 10S diet in this study.

*c:* Based on findings in the present study.

(i.e., rats exposed to BD from pregnancy through PND 50 or lactation through PND 50). As summarized in Table 2, the reduction in the density of TEBs was similar between studies. Moreover, regardless of whether rats were exposed to 10F during pregnancy and lactation only or continuously through PND 50 or during lactation only or continuously through PND 50, the reduction in the density of TEBs was similar, ranging from 66% to 78% of the BD group. Although our previous study did not investigate the effect of exposing rats to a 10S diet, the present study demonstrated that the 10S and the 10F diets reduced the density of TEBs to a similar extent. Also, exposure during lactation only or continuously through PND 50 reduced the density of TEBs by a similar amount.

Figure 2 shows the typical appearance of whole-mount mammary gland preparations with high levels of TEBs and low levels of ABs (BD-BD group) and with low levels of TEBs and high levels of ABs (10S-BD group).

Discussion

This study is the first to demonstrate that exposure to 10% flaxseed or the equivalent amount of purified lignan during lactation only or from lactation through PND 50 can reduce the TEB structures in the mammary gland. This study has also identified that it is the lignans in flaxseed that mediate the maturation of the mammary gland, inasmuch as exposure to the 10S and 10F diets reduced the density of TEBs to a similar extent. Corresponding elevations in the density of ABs were observed among rats receiving the 10F or 10S diet, indicating that the reduction in the density of TEBs was due to the enhanced differentiation of TEBs to ABs. These changes in mammary gland structures, particularly the reduction in the density of TEB structures, may translate into chemopreventive effects, inasmuch as the TEBs are most susceptible to carcinogens (6).

It is also important to consider that there is evidence that postlactational exposure to the 10F diet, and particularly to the 10S diet, further promotes the differentiation of mammary gland structures. We previously observed and reported that rats that are not exposed to flaxseed during pregnancy and/or lactation but are fed flaxseed from PND 21 through PND 50 experience marginal but nonsignificant alterations in the numbers of TEBs and ABs (9). Although the density of lobules was higher among rats continuously exposed to the 10S diet, this difference was <0.3 lobules/mm² among the group with the lowest (BD-BD) and highest density of lobules (10S-10S). Moreover, the lobule density of all groups was small. The reason continuous exposure to the 10S and not the 10F diet resulted in a higher density of lobules than exposure to BD is unclear and requires further verification, since the intakes of SDG were similar among groups, and differences between the bioavailability of flaxseed and purified lignan have not been studied at the earliest stages of the life cycle. Together, these findings emphasize the fact that lactation is a critical stage of mammary gland development that can be manipulated by dietary components to reduce the potential risk of developing mammary cancer.

When we compare the findings of the present study and our former study (9), similar changes in the density of TEBs and ABs are observed. Thus, whether rats were exposed to the 10F diet during pregnancy and lactation or only during lactation, reductions in the density of TEBs with corresponding elevations in the density of ABs are observed.

Figure 2. A typical representation of distal region of mammary glands (abdominal, Gland 4) from rats continuously exposed to BD (A) or exposed to 10F or 10S diet during lactation only or continuously from lactation through PND 50 (B). Note predominance of TEBs (club-shaped structures) in A and decline of these structures accompanied by an elevation of ABs in B. Magnification ×9.
Thus, by deduction, this suggests that lactation is a critical time to enhance the differentiation of TEBs to ABs.

Clarkson and co-workers (17) hypothesized, on the basis of the changes in endogenous levels of circulating estrogen throughout the life cycle, that there is an optimal range of estrogen that is beneficial for overall health. Using this hypothesis and the knowledge that lactation is a hormone-sensitive period, we speculate that exposure to exogenous lignan during early life (i.e., during lactation), when endogenous estrogen levels are low, results in an estrogen-like effect compared with the lesser estrogen-like effect observed among rats exposed to lignan after lactation, when endogenous estrogen levels are higher. This hypothesis makes biological sense in terms of the estrogen-like effects we observed in the mammary glands in this study with the 10F and 10S diets and also in agreement with previous studies in our laboratory that reported that exposure to the 10F diet during pregnancy and lactation resulted in a shorter anogenital distance, an earlier age at puberty onset, and longer estrous cycles due to prolonged estrus (13). All these outcomes are indicators that exposure to lignans before the end of lactation resulted in estrogen-like effects.

The mechanism(s) by which exposure to flaxseed or SDG during lactation enhances the differentiation of TEBs to ABs and lobules remains uncertain and requires investigation in the future. If lignans have an action similar to other phytoestrogens, such as genistein, it is possible that early exposure to lignans reduces the proliferative activity of the TEBs (10–12). It is also possible that lignans may act locally at the mammary gland tissue by altering the level of specific growth factors (i.e., transforming growth factor-α, epidermal growth factor), growth factor receptors, and/or signaling pathways or systemic sex steroid hormones that are mediators of mammary gland development and differentiation (8,18,19). Recently, prepubertal administration of genistein has been reported to enhance the differentiation of TEBs to ABs by increasing levels of transforming growth factor-α and the epidermal growth factor receptor in the mammary tissue (18). These potential mechanisms of action need to be investigated in future studies.

The purpose of this study was to establish whether natural compounds, such as lignans from flaxseed, provided to the offspring during lactation only, altered mammary gland development and differentiation. Thus this study is the first to provide evidence that exposure to flaxseed during lactation can enhance mammary gland development and, thereby, potentially protect against mammary cancer and perhaps breast cancer in humans. Furthermore, this study provides direct evidence that it is the lignan component in flaxseed that mediates these changes in the mammary gland. The next step is to evaluate whether the changes in mammary gland structures resulting from exposure to flaxseed or its purified lignan during lactation do indeed have chemopreventive effects. The tumorigenic response to carcinogen among rats exposed to flaxseed or purified lignan during lactation needs to be studied to confirm whether early diet can potentially protect against the onset of diseases such as breast cancer during adulthood.

Acknowledgments and Notes

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