Exposure to Flaxseed or Its Purified Lignan during Suckling Inhibits Chemically Induced Rat Mammary Tumorigenesis

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Previous studies have shown that feeding flaxseed (FS) or its lignan secoisolariciresinol diglucoside (SDG) to rat dams during lactation enhances the differentiation of rat mammary gland in the female offspring. This study determined whether exposure to a diet with 10% FS or SDG (equivalent to the amount in 10% FS) during suckling could protect against 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced rat mammary tumorigenesis later in life. Dams were fed the AIN-93G basal diet (BD) throughout pregnancy. After delivery, dams were randomized to continue on BD or were fed BD supplemented with 10% FS or SDG during lactation. Three-day urine of dams was analyzed for mammalian lignans. After weaning, all offspring were fed BD. At postnatal Days 49 to 51, during proestrus phase, offspring were gavaged with 5 mg of DMBA. At Week 21 post-DMBA administration, compared with the BD group, the FS and SDG groups had significantly lower ($P < 0.05$) tumor incidence (31.3% and 42.0% lower, respectively), total tumor load (50.8% and 62.5% lower, respectively), mean tumor size (43.9% and 67.7% lower, respectively), and tumor number (46.9% and 44.8% lower, respectively) per rat. There was a significant decreasing trend ($P < 0.05$) in final tumor weights in rats fed FS or SDG. The high urinary lignan excretion in dams fed with FS or SDG corresponded with the reduced tumor development. The FS and SDG groups did not differ significantly in tumor indices, indicating that the effect of FS is primarily due to its SDG. There were no significant changes in selective reproductive indices measured among dams and offspring. In conclusion, exposure to FS or SDG during suckling suppressed DMBA-induced rat mammary tumorigenesis, suggesting that exposure to lignans at this early stage of mammary gland development reduces susceptibility to mammary carcinogenesis later in life without adverse effects on selective reproductive indices in dams or offspring. Exp Biol Med 228:951–958, 2003

Key words: flaxseed; lignan; mammary gland; tumorigenesis

Mammary carcinogenesis has been associated with the development and differentiation of the mammary gland during early life (1). The incidence of carcinomas is positively correlated with the number of highly proliferative and undifferentiated terminal end buds (TEBs) in rodent models at time of carcinogen exposure (2, 3). Thus, an enhancement of mammary gland development by increasing the differentiation of TEBs may be a strategy to protect against mammary tumorigenesis.

Phytoestrogens such as lignans, which exhibit weak estrogenic and antiestrogenic properties in a tissue-specific manner, have potential in the prevention and treatment of breast cancer (4–6). Flaxseed (FS) is the richest source of the plant lignan secoisolariciresinol diglucoside (SDG), which can be metabolized by the colonic microflora to the mammalian lignans, enterodiol (ED) and enterolactone (EL) (7). FS and its lignans have been reported to inhibit chemically induced mammary tumorigenesis at the preinitiation, early, and late promotion stages of carcinogenesis in rats (8–10), and the growth and metastasis of human breast cancer in nude mice (11, 12). However, the prevention of mammary tumorigenesis by exposure to FS lignans at early stage of mammary gland development (i.e., during suckling) has not been demonstrated.

Recent studies in our laboratory have shown that feeding FS or SDG to rat dams during pregnancy and/or lactation promotes maturation of mammary gland in female offspring, resulting in a lower number of highly proliferative TEBs and a higher number of the more differentiated structure, alveolar buds (ABs) (13, 14). It is unknown whether such enhancement of the mammary gland differentiation induced by FS or its lignans can protect the mammary gland against carcinogen-induced tumorigenesis later in life. Reduced risk of mammary tumorigenesis has been observed among rat offspring exposed to soy and/or its isoflavone

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genistein during gestation, suckling, or prepubertal period of female offspring. This effect has been attributed to the early mammary gland development and differentiation enhanced by genistein treatment (15–17). Based on the structural and functional similarity of lignans to genistein and their similar enhancement of early mammary gland development, we hypothesized that exposure to 10% FS, or the equivalent amount of purified SDG present in 10% FS during suckling, could reduce mammary tumorigenesis in the female offspring at adulthood. Therefore, the objective of this study was to determine if exposure to 10% FS or its SDG during suckling period would protect the female offspring against carcinogen-induced mammary tumorigenesis later in life. The potential adverse effects of this exposure on the selected reproductive indices of the dams and offspring were also examined.

Materials and Methods

Diets. The basal diet (BD) was based on the semipurified AIN-93G diet (18). The 10% FS diet was prepared by adding 10% (w/w) ground FS (Linnott Variety; Omega Products, Melfort, Saskatchewan, Canada) to a modified BD that was corrected for macronutrients and calories contributed by FS, as described previously (13, 14). The SDG diet was prepared by adding 20.1 mg of purified SDG/100 g BD, which is equivalent to the amount of SDG in 10% FS as determined by HPLC analysis (19). All diets were prepared by Dyets Inc. (Bethlehem, PA) and were stored at 4°C. Fresh diet was provided to rats every 2 to 3 days. Diet intake was determined by subtracting the food left from food provided at the previous feed. To prevent access of offspring to the diets and to prevent spillage, all diets were given to the dams in tall feeding jars.

Experimental Design. Thirty-six, 8-week-old, timed-pregnant Sprague-Dawley rats (Charles River, Montreal, Quebec, Canada) at Day 7 of gestation were housed in a temperature-controlled facility with a 12:12-hr light/dark cycle. All rats were fed the BD with free access to water for the duration of pregnancy. At the time of delivery, between Days 21 and 22 of gestation, dams were randomly grouped and fed one of three diets, i.e., BD, FS, or SDG (n = 12/group), throughout lactation. At postnatal Day (PND) 3, the number of pups per litter was reduced to eight with five to six females and two to three males. At the end of suckling, when offspring were 21 days old, the female offspring were separated from their mothers and were fed BD throughout the remainder of the study. Thus, offspring were exposed to FS or SDG only during the suckling period. One dam in the BD group had health complications after delivery. Therefore, this dam and her offspring were excluded from the study. All female offspring (n = 47, 54, and 56 for BD, FS, and SDG groups, respectively) were monitored for stage of the estrous cycle daily by a vaginal smear examination starting at PND 40 as described previously (13). When rats were at proestrus phase at PND 49 to 51, a single dose of 5 mg of 9,10-dimethyl-1,2-benzanthracene (DMBA) dissolved in 1.0 ml of corn oil per rat was gavaged to two to four female offspring per litter. The BD (n = 29), FS (n = 38), and SDG (n = 40) groups gavaged with DMBA had mean body weights of 185.2 ± 2.3 g, 186.3 ± 2.6 g, and 185.8 ± 3.1 g, respectively. The other female offspring per litter not treated with DMBA were sacrificed to determine the effect of diet treatment on the toxicity, reproductive organs, and mammary gland development. The male offspring were used in another study.

All rats were code-labeled so that the researchers were blinded to the experimental diets that the rats were exposed to during suckling. The tumor incidence and the two largest perpendicular diameters of established tumor were recorded weekly. The tumor size was calculated using the formula of length/2 × width/2 × π. Rats were sacrificed 21 weeks after DMBA administration (approximately PND 200). The mammary tumors were excised, weighed, and preserved in 10% buffered formalin solution for histological analysis. All major organs were weighed and underwent gross pathological examination. The protocol of this study was approved by the Animal Care Ethics Committee at the University of Toronto. All animal care and procedures were conducted in accordance with the *Guide to the Care and Use of Experimental Animals* (20).

Urinary Lignan Analysis. At the end of lactation, all dams were placed individually in metabolic cages for a 3-day urine collection. The same diets as they received during lactation were given. Daily urine was collected with 1 ml of 0.1% (w/v) ascorbic acid as preservative. Urine collected over 3 days was pooled, centrifuged to remove solid contaminants, and the total urine volume was measured. A 2.5-ml aliquot of urine was analyzed for lignans by capillary gas chromatography-mass spectrometry (GC-MS; GC, model 5890, series II; MS, model 5971; Hewlett-Packard Canada, Mississauga, Ontario, Canada) as described previously (21, 22).

Reproductive Indices and Toxicological Observations in Dams and Offspring. To examine the effect of the dietary exposure on prolactin secretion in rat dams, 200 to 300 μl of blood was obtained from the leg vein of the rat dams at PND 21 when dams were still with their pups and fed their respective treatment diets. The serum was analyzed for prolactin using an enzyme-linked immunoassay kit (Amersham Biosciences, Piscataway, NJ). At postlactation Day (PLD) 3, after urine was collected, one-half of the dams were sacrificed by CO_2 inhalation and their sex and major organs, such as uterus, ovaries, kidney, liver, heart, lungs, adrenal glands, brain, and pituitary, were excised, weighed, and examined for gross pathological changes. The other one-half of the dams were fed BD until PLD 30, during which the length of estrous cycles were determined by daily vaginal smear examination. They were sacrificed at PLD 30 and their organs were examined in the same manner as dams sacrificed at PLD 3.

A number of reproductive indices were measured as described previously (23–25) to assess the hormonal and

toxicological effects of treatments on the offspring. Anogenital distance (AGD), the distance from the genitalia papilla to the anus, was measured at PND 3 and PND 20. The onset of puberty (the visual opening of vaginal aperture), which usually occurs around PND 32 to 38, was determined by daily examination starting at PND 26. Estrous cycle length was measured by daily vaginal smear examination from PND 40 to PND 49 to 51 as described above. At proestrus phase on PND 49 to 51, the remaining rats not gavaged with DMBA were sacrificed by CO_2, and their sex and major organs were excised, weighed, and examined similar to those performed on the dams to examine gross pathological changes.

Statistical Analysis. Data are presented as means ±SEM. Differences in final tumor weights, reproductive indices, and body weight gain and organ weight were determined by one-way analysis of variance (ANOVA) for parametric data or ANOVA on rank for nonparametric data, followed by *post hoc* tests by either Tukey's or Dunn's pair-wise comparison using SigmaStat (Jandel Scientific, San Rafael, CA). The weekly tumor parameters among treatment groups over the palpation period were analyzed by repeated-measures one-way ANOVA with general linear model, followed by Tukey's test (SPSS 10.01 SPSS Inc., Chicago, IL). The tumor incidence over the study period was assessed by log-rank test using SPSS. The final total tumor weight (tumor load) per rat and histological data were stratified and compared by Armitage's trend test in proportions (26). Tumor invasion was analyzed by chi-square test.

Results

Diet Intake and Body Weight Gain in Dams and Offspring. Mean daily diet intakes did not differ among dams during lactation (32.0 ± 1.5 g/rat, 34.9 ± 1.0 g/rat, and 32.0 ± 1.4 g/rat for BD, FS, and SDG groups, respectively) or among offspring after weaning until PND 49 to 51 (9.9 ± 0.3 g/rat, 9.7 ± 0.4 g/rat, and 9.7 ± 0.2 g/rat for BD, FS, and SDG groups, respectively). The body weights of dams at PLD 3 were not significantly different among treatment groups (270.9 ± 9.6 g, 268.3 ± 7.8 g, and 268.9 ± 7.2 g for BD, FS, and SDG group, respectively). There were also no significant differences among treatment groups in body weights of offspring either at DMBA administration (described above) or at necropsy after DMBA administration (349.2 ± 11.1 g, 359.0 ± 9.9 g, and 355.3 ± 10.1 g for BD, FS, and SDG group, respectively).

Daily Urinary Lignan Excretion of Dams. Table I shows the daily urinary lignan excretion in dams fed the different diets. Dams in the BD group excreted negligible amounts of lignans, ED, EL, and secoisolariciresinol (SECO). ED was the major mammalian lignan excreted in rats fed FS and SDG diets. The ED, EL, SECO, and total urinary lignans (ED + EL + SECO) were significantly higher in the FS and SDG groups than BD group. Conversely, the FS group excreted a higher level of total lignans than the SDG group, similar to the observations in our previous studies (21, 22).

Tumor Induction. There was no significant difference in the latency, i.e., mean time for the first tumor appearance post-DMBA administration, among groups (13.3 ± 1.0 weeks, 13.7 ± 0.9 weeks, and 13.8 ± 1.0 weeks for the BD, FS, and SDG groups, respectively). However, a significantly lower tumor incidence was consistently observed in both the FS and SDG groups, with a 31.3% and 42.0% reduction, respectively, compared with the BD group at Week 21 ($P < 0.05$; Fig. 1).

The palpable tumor number, tumor load, and mean tumor size per rat in group were consistently affected by the treatments during suckling. The mean tumor number per rat (Fig. 2) was significantly lower ($P < 0.05$) in rats fed FS and SDG starting at Weeks 14 and 12, respectively. At the end of study (Week 21), 46.9% and 44.8% lower tumor number per rat was observed in the FS and SDG groups, respectively, compared with the BD control. Starting at Week 10, the FS and SDG groups had significantly lower ($P < 0.05$) tumor load per rat than the BD group (Fig. 3). By Week 21, significant reductions ($P < 0.05$) of 50.8% and 62.5% were induced by FS and SDG, respectively, compared with the control (Fig. 3). The mean tumor size per rat was also significantly lower in the FS and SDG groups starting at Week 10 after DMBA administration, with 43.9% and 67.7% lower values, respectively, by Week 21 (Fig. 4)

At necropsy, the final total tumor weight per tumor bearing rat in the FS (4.09 ± 1.35 g) and SDG (2.80 ± 0.88 g) groups were lower than that in the BD group (5.94 ± 2.19 g), although the differences were not statistically significant. However, when rats were classified into four categories of total tumor loads (weights) per rat, a significant trend for lower tumor load ($P < 0.05$) was observed in the FS and SDG groups versus the BD group, but not between FS and SDG groups (Table II).

Table I. Effect of Exposure to 10% FS and the Equivalent Amount of SDG during Lactation on Daily Urinary Lignan Excretion in Dams

	n	ED (μM/day)	EL (μM/day)	SECO (μM/day)	Total (μM/day)
BD	11	0.02 ± 0.00^a	0.03 ± 0.00^a	ND	0.05 ± 0.01^a
FS	12	10.52 ± 1.43^b	0.40 ± 0.05^b	0.13 ± 0.02^a	11.04 ± 1.43^b
SDG	12	2.42 ± 0.35^c	0.18 ± 0.03^b	0.40 ± 0.07^b	2.99 ± 0.33^c

Note. Data are means ± SEM. Different superscripts within a column denotes a significant difference at $P < 0.05$ by one-way ANOVA. BD, basal diet; ED, enterodiol; EL, enterolactone; SECO, secoisolariciresinol; ND, not detectable.

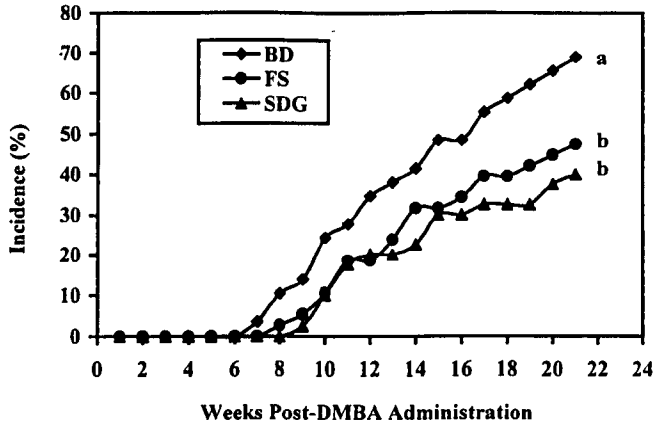

Figure 1. Palpable mammary tumor incidence (%) in female Sprague-Dawley rats exposed to FS or SDG during suckling. Immediately after delivery, lactating dams were randomized to either continue on basal diet (BD, n = 11) or to a diet supplemented with 10% FS (n = 12) or with SDG (n = 12). After weaning, all offspring (n = 29, 38, and 40 per group for BD, FS, and SDG, respectively) were fed with BD only, and were gavaged with 5 mg of DMBA on PND 49 to 51 at proestrus phase. The palpable tumors were monitored weekly until Week 21. Different letters indicate a significant difference ($P < 0.05$) among groups by log-rank test throughout the study starting at Week 12.

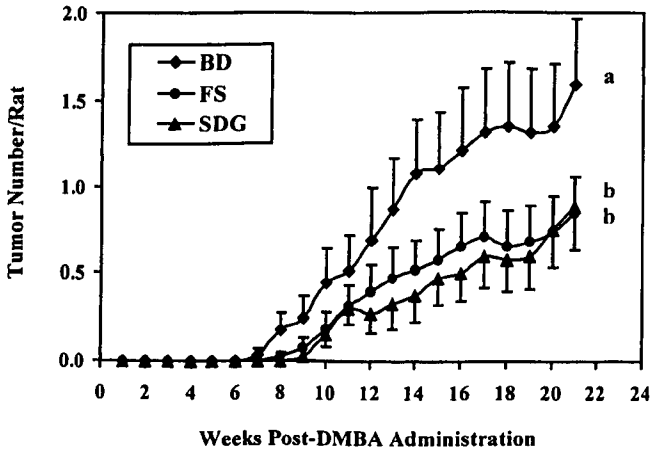

Figure 2. Mammary tumor number per group in female Sprague-Dawley rats exposed to FS or SDG during suckling. Weekly values are group means with error bar (SEM). Immediately after delivery, lactating dams were randomized to either continue on basal diet (BD; n = 11) or to a diet supplemented with 10% FS (n = 12) or with SDG (n = 12). After weaning, all offspring (n = 29, 38, and 40 for BD, FS, and SDG groups, respectively) were fed with BD only, and were gavaged with 5 mg of DMBA on PND 49 to 51 at proestrus phase. The palpable tumors were monitored weekly until Week 21. Different letters indicate a significant difference ($P < 0.05$) in tumor number per rat among groups throughout the study period (by general linear model of one-way ANOVA repeated measures followed by Tukey's test) starting at Week 14 for BD versus FS and at Week 12 for BD versus SDG.

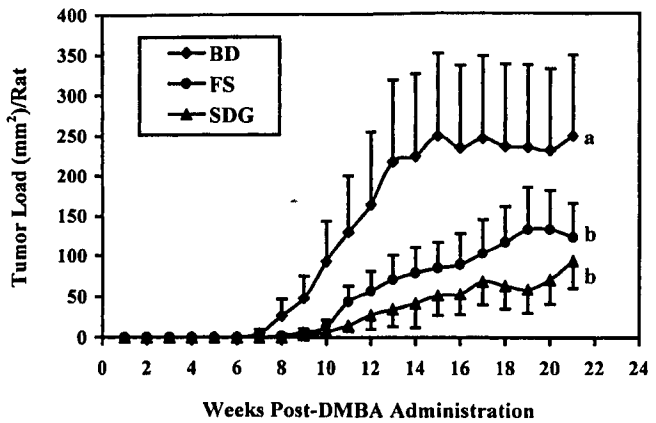

Figure 3. Total mammary tumor load (millimeters squared) per group in female Sprague-Dawley rats exposed to FS or SDG during suckling . Weekly values are group means with error bar (SEM). Immediately after delivery, lactating dams were randomized to either continue on basal diet (BD; n = 11) or to a diet supplemented with 10% FS (n = 12) or with SDG (n = 12). After weaning, all offspring (n = 29, 38, and 40 for BD, FS, and SDG groups, respectively) were fed with BD only, and were gavaged with 5 mg of DMBA on PND 49 to 51 at proestrus phase. The palpable tumors were monitored weekly until Week 21. Different letters indicate a significant difference ($P < 0.05$) in total tumor load per rat among groups (measured by general linear model of one-way ANOVA repeated measures followed by Tukey's test) starting at Week 10 for BD versus FS or SDG.

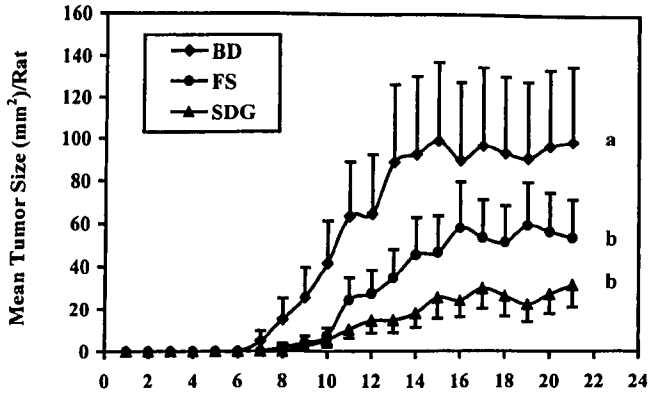

Figure 4. Mean mammary tumor size (millimeters squared) per group in female Sprague-Dawley rats exposed to FS or SDG during suckling. Weekly values are group means with error bar (SEM). Immediately after delivery, lactating dams were randomized to either continue on basal diet (BD; n = 11) or to a diet supplemented with 10% FS (n = 12) or with SDG (n = 12). After weaning, all offspring (n = 29, 38, and 40 for BD, FS, and SDG groups, respectively) were fed with BD only, and were gavaged with 5 mg of DMBA on PND 49 to 51 at proestrus phase. The palpable tumors were monitored weekly until Week 21. Different letters indicate a significant difference ($P < 0.05$) in mean tumor size per rat among groups (measured by general linear model of one-way ANOVA repeated measures followed by Tukey's test) starting at Week 10 for BD versus FS or SDG.

Histopathological analysis did not reveal significant differences in the ratio of benign to malignant tumors or in histological grades among groups. However, no invasion to the surrounding tissues, such as skeletal muscle, lymph nodes, and parotid gland, by the primary tumor was found in the SDG group compared with the BD and FS groups. Hence, the SDG group differed significantly ($P < 0.05$) from the BD and FS groups in tumor invasion. The invasion was seen only in large tumors with higher grade in the BD and FS groups.

Toxicity and Reproductive Index Assessments in Dams and Offspring. Table III presents the serum prolactin level at the end of lactation and estrous cycle

Table II. Effect of Exposure to 10% FS or the Equivalent Amount of SDG during Suckling on Total Tumor Weight and Histological Grade of Offspring at Necropsy

	Number of rats (%) with following total tumor weight[a]					Histological analysis						
						Number of tumors analyzed	Number of benign tumors (%)	Number of adenocarcinomas (%)				
								Grade (%)[c]			Invasion (%)[d]	
	n^b	0 g	<1.0 g	1.0–10.0 g	>10.0 g			I	II	III	–	+
BD	29	9 (31.0)	7 (24.1)	9 (31.0)	4 (13.8)	45	7 (15.6)	12 (31.6)	20 (52.6)	6 (15.8)	31 (81.6)	7 (18.4)
FS	38	20 (52.6)	10 (26.3)	5 (13.2)	3 (7.9)	34	6 (17.7)	12 (42.9)	13 (64.4)	3 (10.7)	24 (85.7)	4 (14.3)
SDG	40	24 (60.0)	7 (17.5)	8 (20.0)	1 (2.5)	34	4 (11.8)	15 (50.0)	13 (33.3)	2 (6.67)	30 (100.0)	0 (0)

[a] Total tumor weight (tumor load) per rat was stratified into four categories and was analyzed by Armitage's trend in proportion. BD versus FS, $P < 0.05$; BD versus SDG, $P < 0.05$; FS versus SDG, $P > 0.05$.
[b] Number of rats gavaged with DMBA.
[c] Histological grade: I, well differentiated; II, moderately differentiated; III, poorly differentiated. No significant difference among groups.
[d] –, no invasion; +, invasive to neighboring tissues, e.g., skeletal muscle, lymph node, and parotid gland. SDG versus BD or FS, $P < 0.05$ (chi-square test).

Table III. Effect of Exposure to 10% FS and the Equivalent Amount of SDG during Lactation on Estrous Cycle Length, Serum Prolactin, and Relative Uterus and Ovary Weights in Dams

	BD	FS	SDG
Estrous cycle length (day)	3.9 ± 0.1	4.1 ± 0.2	4.4 ± 0.3
	$n = 5$	$n = 6$	$n = 6$
Serum prolactin at PND21 (ng/ml)	25.1 ± 3.5	24.8 ± 4.2	21.3 ± 3.5
	$n = 11$	$n = 12$	$n = 12$
PLD 3	$n = 6$	$n = 6$	$n = 6$
Relative ovary weight (mg/100 g body weight)	62 ± 7	58 ± 4	60 ± 2
Relative uterus weight (mg/100 g body weight)	208 ± 31	168 ± 17	177 ± 26
PLD 30	$n = 5$	$n = 6$	$n = 6$
Relative ovary weight (mg/100 g body weight)	53 ± 3	53 ± 3	52 ± 2
Relative uterus weight (mg/100 g body weight)	170 ± 17	176 ± 18	184 ± 18

Note. Values are means ± SEM. No significant difference among groups. BD, basal diet; PND, postnatal day; PLD, postlactation day.

length after lactation in dams. Both were not affected by treatments. The relative weights of uterus and ovaries at PLD 3 and 30 were also not significantly different among groups.

In the offspring, the relative changes in AGD were not significantly different among groups (Table IV). The time of puberty onset, estrous cycle length, and relative weights of uterus and ovaries at PND 21 and 49 to 51 were similar in all groups (Table IV).

Weights of other major organs (i.e., liver, kidney, brain, and lungs) relative to body weight were also similar and not significantly different among groups in the dams (at PLD 3 and 30) and offspring (at PND 21 and 49 to 51; data not shown).

Discussion

This study demonstrated, for the first time, that exposure to FS or its equivalent amount of SDG during suckling significantly reduces mammary tumorigenesis in rats later in life. This supports our hypothesis that enhanced development and differentiation of mammary gland induced by FS and its lignans during early life can reduce the risk of mammary tumorigenesis in rats at adulthood.

It has been suggested that early development of mammary gland is an important determinant of tumorigenesis later in life, and early exposure to estrogen plays a critical role in the development of mammary gland (1). At prepubertal stage of rodents, the mammary gland consists mostly of TEBs, which are highly proliferative, the least mature, and the most susceptible structure to carcinogens. With increasing estrogen levels at puberty, TEBs differentiate to ABs, the basic structure of lobules, which are less proliferative and more resistant to carcinogens (2, 3). Our previous studies have shown that exposure of female rats to either FS or SDG during gestation and suckling (13) or suckling alone (14) resulted in decreased TEBs and higher ABs in the mammary gland of the offspring at PND 50. Furthermore, exposure to FS or SDG during the suckling period only (14) resulted in the same effect as exposure during gestation and suckling (13), indicating that the critical time of exposure for mammary gland cell differentiation occurs mainly during suckling. In the present study, a similar pattern of mammary gland development was observed (27), where a differentiation effect induced by FS and SDG in early development of mammary gland resulted in lower TEBs and higher ABs and lobules. This chemopreventive

Table IV. Effect of Exposure to 10% FS and the Equivalent Amount of SDG during Suckling on Selective Reproductive Indices in Female Offspring

	BD	FS	SDG
	n = 55	*n* = 68	*n* = 70
Relative change in AGD[a] (mm/100 g)	16.65 ± 0.14	15.67 ± 0.14	16.17 ± 0.21
Puberty onset (PND)	33.5 ± 0.4	33.6 ± 0.3	32.5 ± 0.3
Estrous cycle length (day)	4.3 ± 0.1	4.3 ± 0.1	4.4 ± 0.1
PND 21	*n* = 12	*n* = 14	*n* = 14
Body weight (g)	47.7 ± 0.7	49.4 ± 1.0	47.3 ± 1.8
Relative ovary weight (mg/100 g body weight)	6 ± 0	6 ± 0	7 ± 0
Relative uterus weight (mg/100 g body weight)	74 ± 4	72 ± 4	80 ± 4
PND 49–51	*n* = 14	*n* = 16	*n* = 16
Body weight (g)	179.5 ± 4.9	182.3 ± 3.7	183.7 ± 5.0
Relative ovary weight (mg/100 g body weight)	62 ± 2	60 ± 2	60 ± 2
Relative uterus weight (mg/100 g body weight)	273 ± 23	258 ± 18	284 ± 21

Note. Values are means ± SEM. No significant difference among groups. BD, basal diet; AGD, anogenital distance; PND, postnatal day.
[a] [(AGD at PND 20) – (AGD at PND 3)]/[(body weight at PND 20) – (body weight at PND 3)].

effect of enhanced glandular differentiation by FS and its lignan SDG is similar to the suggested protection from early full-term pregnancy in humans (28, 29), whereby hormonal exposure results in earlier glandular maturity.

The mechanism(s) by which early exposure to FS and its lignans enhances maturation of rat mammary gland is not clear and requires further study. Mammalian lignans are biphenolic compounds, and their weak estrogenicity may be related to their structural similarity to estrogen (4). Previous studies observed that exposure to FS or lignan early in life, i.e., gestation and suckling periods, when endogenous estrogen levels are low, results in an estrogen-like effect in the development of reproductive system (23, 24). Furthermore, elevated serum levels of estradiol were detected in the offspring exposed to 10% FS or SDG from gestation through the end of suckling (23, 24), which may stimulate mammary gland development. It is possible that lignans may also act at the mammary gland tissue by modulating the signal transduction pathway. It has been reported that exposure to the phytoestrogen genistein during development of mammary gland modulates the expression of specific growth factors, such as transforming growth factor-α, epidermal growth factor (EGF), and EGF receptor (EGFR) in the mammary gland (30). This action may be through regulation of the expression of estrogen receptor-α and progesterone receptor by genistein (31). In our previous studies, plasma insulin-like growth factor-I level in rats (32) and expression of insulin-like growth factor-I, EGFR, and vascular endothelial growth factor in transplanted human breast tumor tissues in nude mice (11, 12) were downregulated by FS or SDG. Further studies are needed to determine whether FS and lignans can similarly modulate these growth factors and receptors in facilitating early development of mammary gland.

It is noteworthy that in the present and previous studies, exposure to FS and SDG during lactation induced significant changes in mammary gland development, but not in selective reproductive indices of offspring (14, 25, 27), whereas exposure to the same components for a longer period, i.e., during gestation and suckling, significantly induced changes in AGD and other hormone-sensitive indices such as relative weights of uterus and ovaries (23, 24). The mechanism underlying these different effects remains unclear. It may be that mammary tissue has greater sensitivity to lignans than other tissues at this critical period. The timing (stage of development), dose, and duration of exposure to phytoestrogen lignans are all important determinants of physiological outcomes as has been reported in our previous studies (13, 14, 23–25). To elucidate the probable mechanistic pathway, an intensive study is currently being conducted in our laboratory to assess the expression of both EGFR and estrogen receptor signaling in the mammary gland.

Using radiolabeled SDG, we previously reported that lignans given to dams during lactation can be transferred to the nursing offspring via milk (23). Exposure to FS or SDG resulted in higher urinary excretion of mammalian lignans, as an indicator of SDG metabolism to ED and EL in the dams, and lower tumorigenesis in offspring in the FS and SDG groups This suggests that lignans may be the major component responsible for the protection against mammary tumorigenesis. Although the urinary lignan level detected in dams fed SDG was lower than that fed the FS, their protective effects were not significantly different except in the case of tumor invasiveness, indicating that only a low amount of lignans is necessary for mammary cancer chemoprevention. The urinary ED and EL levels are higher in the FS group than that in the SDG group, but the level of SECO, the aglycone of SDG, in the urine of the SDG group was significantly higher than that in the FS group. It is currently unknown whether the high SECO level or the difference in the ED to EL ratio was responsible for the small difference in the effect of SDG versus FS. The possible explanations for a lower urinary excretion of lignans in the SDG group than that in the FS group may be related to the fact that FS also contains other mammalian lignan precursors, such as matairesinol and pinoresinol, which may contribute to a greater mammalian lignan production in the

body (33, 34); other components in FS, such as dietary fibers, may facilitate colonic fermentation, resulting in an increased mammalian lignan production from the precursors (35, 36); or the HPLC method used in the SDG analysis underestimated the amount of SDG present in FS. Nevertheless, the relationship of high urinary lignan excretion with lower tumor induction in the present study is supportive of the findings in epidemiological case control studies, which showed that cancer risk is lower in the population with higher urinary lignan excretion (37, 38).

FS is also rich in α-linolenic acid (ALA), an ω-3 fatty acid that has been reported to have anticancer effects in established rat mammary tumors (9). However, the tumor inhibitory effects of FS and SDG in the present study are similar, suggesting that the tumor protective effect of FS is largely contributed by lignans and not by ALA. Furthermore, previous studies did not reveal any effect of ALA in FS on the early development of mammary gland (13).

This study also demonstrated, for the first time, no obvious adverse effect on rat dams consuming FS and its lignans during lactation. Our findings that there were no gross adverse effects on female offspring exposed to FS or its lignans during suckling are in agreement with observations in our previous studies (14, 25). Although the present and past studies (14, 25) suggest that FS consumption by lactating mothers may protect the offspring from developing mammary cancer without negative effects on selective reproductive and toxicological indices, extensive human clinical studies are needed before definitive conclusion on their safety can be made. The intake of 5% to 10% FS by rats is equivalent to 25 to 50 g/day in humans, depending on their total food intake.

In conclusion, this study provides the first experimental evidence that exposure of rat offspring during suckling to 10% FS or its equivalent level of lignan SDG inhibits DMBA-induced mammary tumorigenesis, while causing no gross toxicity and adverse effect on selective reproductive indices in dams and offspring. The mammary chemoprotective effect of FS appears to be primarily due to its lignans. The results from this study support the hypothesis that exposure to estrogen-like compounds such as phytoestrogens at an early stage of mammary gland development may reduce breast cancer risk at adulthood without causing severe adverse effects.

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1. Russo J, Lynch H, Russo IH. Mammary gland architecture as a determining factor in the susceptibility of the human breast to cancer. Breast J **7**:278–291, 2001.
2. Russo IH, Russo J. Developmental stage of the rat mammary gland as determinant of its susceptibility to 7,12-dimethylbenz (a) anthracene. J Natl Cancer Inst **61**:1439–1449, 1978.
3. Russo J, Russo IH. DNA labeling index and structure of the rat mammary gland as determinant of its susceptibility to carcinogenesis. J Natl Cancer Inst **61**:1451–1459, 1978.
4. Thompson LU. Flaxseed, lignans, and cancer. In: Cunnane SC, Thompson LU, Eds. Flaxseed in Human Nutrition. Champaign, IL: AOCS Press, pp219–236, 1995.
5. Thompson LU. Experimental studies on lignans and cancer. Baillieres' Clin Endocrinol Metab **12**:691–705, 1998.
6. Thompson LU, Ward WE. Flaxseed lignans: health benefits, bioavailability, and safety. In: Gilani GS, Anderson JB, Eds. Phytoestrogens and Health. Champaign, IL: AOCS Press, pp405–426, 2002.
7. Thompson LU, Robb P, Serraino M, Cheung F. Mammalian lignan production from various foods. Nutr Cancer **16**:43–52, 1991.
8. Serraino M, Thompson LU. The effect of flaxseed supplementation on the initiation and promotional stages of mammary tumorigenesis. Nutr Cancer **17**:153–159, 1992.
9. Thompson LU, Rickard SE, Orcheson LJ, Seidl MM. Flaxseed and its lignan and oil components reduce mammary tumor growth at a late stage of carcinogenesis. Carcinogenesis **17**:1373–1376, 1996.
10. Thompson LU, Seidl MM, Rickard SE, Orcheson LJ, Fong HH. Antitumorigenic effect of a mammalian lignan precursor from flaxseed. Nutr Cancer **26**:159–165, 1996.
11. Chen J, Stavro PM, Thompson LU. Dietary flaxseed inhibits human breast cancer growth and metastasis and downregulates expression of insulin-like growth factor and epidermal growth factor receptor. Nutr Cancer **43**:187–192, 2002.
12. Dabrosin C, Chen J, Wang L, Thompson LU. Flaxseed inhibits metastasis and decreases extracellular vascular endothelial growth factor in human breast cancer xenografts. Cancer Lett **185**:31–37, 2002.
13. Tou JC, Thompson LU. Exposure to flaxseed or its lignan component during different developmental stages influences rat mammary gland structures. Carcinogenesis **20**:1831–1835, 1999.
14. Ward WE, Jiang FO, Thompson LU. Exposure to flaxseed or purified lignan during lactation influences rat mammary gland structures. Nutr Cancer **37**:187–192, 2000.
15. Lamartiniere CA, Murrill WB, Manzolillo PA, Zhang JX, Barnes S, Zhang X, Wei H, Brown NM. Genistein alters the ontogeny of mammary gland development and protects against chemically induced mammary cancer in rats. Proc Soc Exp Biol Med **217**:358–364, 1998.
16. Murrill WB, Brown NM, Zhang JX, Manzolillo PA, Barnes S, Lamartiniere CA. Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. Carcinogenesis **17**:1451–1457, 1996.
17. Hilakivi-Clarke L, Onojafe I, Raygada M, Cho E, Skaar T, Russo I, Clarke R. Prepubertal exposure to zearalenone or genistein reduces mammary tumorigenesis. Br J Cancer **80**:1682–1688, 1999.
18. Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr **123**:1939–1951, 1993.
19. Obermeyer WR, Musser SM, Betz JM, Casey RE, Pohland AE, Page SW. Chemical studies of phytoestrogens and related compounds in dietary supplements: flax and chaparral. Proc Soc Exp Biol Med **208**:6–12, 1995.
20. Canadian Council on Animal Care. Guide to the Care and Use of Experimental Animals. Canadian Council on Animal Care. Ottawa, Ontario, Canada, 1984.
21. Rickard SE, Orcheson LJ, Seidl MM, Luyengi L, Fong HHS, Thompson LU. Dose-dependent production of mammalian lignans in rats and in vitro from the purified precursor secoisolariciresinol diglycoside in flaxseed. J Nutr **126**:2012–2019, 1996.
22. Rickard SE, Thompson LU. Urinary composition and postprandial blood changes in H-secoisolariciresinol diglycoside (SDG) metabolites in rats do not differ between acute and chronic SDG treatments. J Nutr **130**:2299–2305, 2000.

23. Tou JC, Chen J, Thompson LU. Flaxseed and its lignan precursor, secoisolariciresinol diglycoside, affect pregnancy outcome and reproductive development in rats. J Nutr **128:**1861–1868, 1998.
24. Tou JC, Chen J, Thompson LU. Dose, timing, and duration of flaxseed exposure affect reproductive indices and sex hormone levels in rats. J Toxicol Environ Health A **56:**555–570, 1999.
25. Ward WE, Chen J, Thompson LU. Exposure to flaxseed or its purified lignan during suckling only or continuously does not alter reproductive indices in male and female offspring. J Toxicol Environ Health A **64:**567–577, 2001.
26. Armitage P. Tests for linear trends in proportions and frequencies. Biometrics **11:**375–386, 1955.
27. Tan KP, Chen J, Thompson LU. Early mammary morphogenesis, reproductive indices, and splenocyte blastogenesis in rats exposed to flaxseed and secoisolariciresinol diglycoside (SDG) during suckling period. FASEB J **16:**A742, 2002.
28. MacMahon B, Cole P, Lin TM, Lower CR, Mirra AP, Ravnihar B, Salber EJ, Valaoras VG, Yuasa S. Age at first birth and breast cancer risk. Bull W.H.O. **43:**209–221, 1970.
29. Lambe M, Hsieh CC, Chan HW, Ekbom A, Trichopoulos D, Adami HO. Parity, age at first and last birth, and risk of breast cancer: a population-based study in Sweden. Breast Cancer Res Treat **38:**305–311, 1996.
30. Brown NM, Wang J, Cotroneo MS, Zhao YX, Lamartiniere CA. Prepubertal genistein treatment modulates TGF-α, EGF and EGF-receptor mRNAs and proteins in the rat mammary gland. Mol Cell Endocrinol **144:**149–165, 1998.
31. Cotroneo MS, Wang J, Fritz WA, Eltoum IE, Lamartiniere CA. Genistein action in the prepubertal mammary gland in a chemoprevention model. Carcinogenesis **23:**1467–1474, 2002.
32. Rickard SE, Yuan YV, Thompson LU. Plasma insulin-like growth factor I levels in rats are reduced by dietary supplementation of flaxseed or its lignan secoisolariciresinol diglycoside. Cancer Lett **161:**47–55, 2000.
33. Meagher LP, Beecher GR, Flanagan VP, Li BW. Isolation and characterization of the lignans isolariciresinol and pinoresinol in flaxseed meal. J Agric Food Chem **47:**3173–3180, 1999.
34. Heinonen S, Nurmi T, Liukkonen K, Poutanen K, Wahala K, Deyama T, Nishibe S, Adlercreutz H. In vitro metabolism of plant lignans: new precursors of mammalian lignans enterolactone and enterodiol. J Agric Food Chem **49:**3178–3186, 2001.
35. Folino M, McIntyre A, Young GP. Dietary fibers differ in their effects on large bowel epithelial proliferation and fecal fermentation-dependent events in rats. J Nutr **125:**1521–1528, 1995.
36. Rowland IR, Wiseman H, Sanders TA, Adlercreutz H, Bowey EA. Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora. Nutr Cancer **36:**27–32, 2000.
37. Ingram D, Sanders K, Kolybaba M, Lopez D. Case-control study of phyto-oestrogens and breast cancer. Lancet **350:**990–994, 1997.
38. Pietinen P, Stumpf K, Mannisto S, Kataja V, Uusitupa M, Adlercreutz H. Serum enterolactone and risk of breast cancer: a case-control study in eastern Finland. Cancer Epidemiol Biomarkers Prev **10:**339–344, 2001.