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Effects of flaxseed lignan secoisolariciresinol diglucoside on preneoplastic biomarkers of cancer progression in a model of simultaneous breast and ovarian cancer development

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Abstract

Breast cancer prevention efforts are focused increasingly on potentially beneficial dietary modifications due to their ease of implementation and wide acceptance. Secoisolariciresinol diglucoside (SDG) is a lignan found in high concentration in flaxseed that may have selective estrogen receptor modulator (SERM)-like effects resulting in antiestrogenic activity in a high estrogen environment. In parallel with a human phase II prevention trial, female ACI rats (n=8–10/group) received 0, 10 or 100 ppm SDG in the feed. The 100 ppm SDG treatment produced similar blood lignan levels as those observed in our human pilot study. Mammary and ovarian cancer progression were induced using local ovarian DMBA treatment and subcutaneous sustained release 17 β -estradiol administered starting at 7 weeks of age. Mammary gland and ovarian tissues were collected at 3 months after initiation of treatment and examined for changes in epithelial cell proliferation (Ki-67, cell counts), histopathology and dysplasia scores as well as expression of selected genes involved in proliferation, estrogen signaling and cell adhesion. Treatment with SDG normalized several biomarkers in mammary gland tissue (dysplasia, cell number, and expression of several genes) that had been altered by carcinogen. There is no indication that SDG promotes pre-neoplastic progression in the ovarian epithelium.

Keywords

mammary; ovary; cancer; flaxseed; lignan; prevention

Introduction

Flaxseed has been the focus of much study as a bioactive dietary component and contains several lipids and lignans suggested to impact risk for hormone-dependent cancers (1, 2). Secoisolariciresinol diglucoside (SDG) is a commercially available component of flaxseed hypothesized to mitigate breast cancer risk through SERM-like actions. SDG is metabolized in the gut to bioactive metabolites enterolactone and enterodiol, both of which can alter estrogen signaling (3, 4). Human epidemiological data, and some preclinical studies, show decreased mammary tumor incidence associated with flaxseed or SDG intake (1, 5). Preclinical studies have reported lengthening of the estrous cycle and reduction in serum IGF-1 with SDG administration suggesting that reduction in hormone signaling and mitogen availability may be partly responsible for antineoplastic actions of the lignan (2, 4). Recent work using the laying hen model of ovarian epithelial carcinoma suggests that flaxseed reduces the progression but not incidence of this hormone-dependent cancer (6), however, others have suggested that SERMs and their mimics can promote ovarian dysplasia (7).

Breast and ovarian cancer share many risk factors, and women at increased risk for breast cancer are also often at increased risk for ovarian cancer (8–10). However, clinical ovarian cancer prevention trials are hampered both by lower disease prevalence as well as the relative inaccessibility of initiated but still benign ovarian tissue for repeated sampling before and after a prevention intervention in early phase trials that use biomarkers as endpoints (8). We have developed an animal model of induced breast and ovarian dysplasia and neoplasia (11, 12) as a proof of principle for simultaneous breast and ovarian cancer prevention, and to address interactions between these related cancers during prevention and carcinogenesis. We report here the initial results of two doses of SDG for effects on mammary and ovarian dysplasia and proliferation as well as biomarker gene expression in this model in parallel with our current human breast cancer chemoprevention trial, following on a successful pilot study (13).

Materials and methods

Animals and treatments

Female ACI rats (Harlan Breeding Laboratories, Indianapolis, IN, n = 8–10 per treatment group) initially weighing 50–55 g were housed in pairs using forced air rat microisolators in a climate and light (12L:12D) controlled environment and received food and water *ad libitum*. All experimental protocols were approved by the University of Kansas Medical Center Animal Care and Use Committee. Hemiovariectomy was performed aseptically in order to concentrate ovulation upon the remaining ovary (14, 15) - a risk factor of human ovarian cancer (11, 16). The remaining ovary was treated by passing a DMBA-impregnated suture (2.5 mm region dipped in DMBA solution) through the ovary twice such that the DMBA or vehicle region was gently secured to the ovarian surface epithelium. Such local ovarian DMBA treatment results in successive ovarian preneoplasia and adenocarcinoma in the majority of rats as described previously by our group and others (11, 12, 17). Rats receiving ovarian DMBA were subsequently treated with sustained release 17 β -estradiol (E₂, 0.75 mg, pellet implant, Innovative Research of America, Sarasota, FL) (18) Our laboratory has previously shown that this treatment combination promotes persistent estrus

and progression toward simultaneous mammary and ovarian cancer in the rat, with significant preneoplastic changes by 3 months of treatment (11) and mammary and ovarian tumors by 6–9 months. SDG was provided in feed ad libitum (Harlan Laboratories) at 0, 10 and 100 ppm SDG to achieve approximate doses of 0, 1 and 10 mg/kg/d estimating intake at 10 g feed/100g BW/d (19). In consultation with a staff nutritionist familiar with the study, we used a modification of the purified diet Teklad TD.94048 (AIN-93M) replacing soybean oil with corn oil.

In addition to these carcinogen and carcinogen + SDG groups, age matched untreated rats were included (n=6) as procedural (naïve) controls.

Serum concentrations of SDG metabolites, 17 β -estradiol and progesterone—

Sera were collected from a subset of rats (n=4 per group) at 3 weeks post-surgery/SDG initiation and stored at –80°C until assay. SDG metabolites secoisolariciresinol, enterolactone, and enterodiol were measured in 100 μ l of rat sera by HPLC coupled to electrospray tandem mass spectrometry (ESI-MS/MS) at Cincinnati Children's Hospital Medical Center via the method of Setchell et al., (20, 21). Serum concentrations (3 month samples) of estradiol and progesterone were measured at the Ligand Assay and Analysis Core in the Center for Research in Reproduction at the University of Virginia using ELISA kits (Estradiol EIA, DSL-10-4300; Progesterone EIA, DSL-10-3900; Diagnostic Systems Laboratories, In., Texas, USA).

Tissue preparation

Rats were killed at 3 months post-treatment and serum was collected and stored at –80°C. Right thoracic mammary glands were excised, fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Right abdominal mammary glands were spread onto a glass slide, fixed in 4% PFA overnight, hydrated, infused with alum carmine (3–4 days), dehydrated, precleared in xylene, and stored in methyl salicylate. Left mammary glands were snap-frozen and stored at –80°C for RNA endpoints. The ovary was bisected through the site of DMBA application. One half was fixed in 4% PFA and embedded in paraffin while the remainder was snap frozen.

Immunohistochemistry

Eight-micron sections of mammary glands and ovaries (n=8–10/group) were deparaffinized, rehydrated, and stained with hematoxylin and eosin. Mammary and ovarian sections (midsagittal, at two different equidistant levels per tissue) were evaluated for morphological changes associated with early mammary adenocarcinoma and epithelial ovarian cancer progression while blinded to treatment group identity (17, 22). Adjacent sections were prepared for immunostaining by antigen retrieval (90°C, 10 mM citrate buffer, 20 minutes) and incubation with 0.3% hydrogen peroxide (Lab Vision, Fremont, CA). Non-immune serum or primary antibody against Ki-67 (1:50; Clone MIB-5; mouse monoclonal antibody, DAKO) was applied and visualized with diaminobenzidine (DAB) chromogen and biotinylated secondary antibody (DAKO). All incubations were carried using a Dako Autostainer Plus (Carpinteria, CA). Ki-67 expression in the mammary ductal epithelia cells

and ovarian surface epithelium was quantified as number of Ki-67 positive (immunoreactive) cells per 500 total epithelial cells counted.

Quantitative Analysis of Preneoplastic Lesions

Sections from equidistant midsagittal areas of the mammary gland were evaluated and assigned a dysplasia score according to the presence of pre-neoplastic and neoplastic lesions associated with breast cancer progression (11, 23). A score of 0 represented normal mammary histology. Preneoplastic changes included mild (score = 1) or severe (2) ductal hyperplasia and/or hyperplasia with atypia (3). Neoplastic changes included ductal carcinoma *in situ* (DCIS, score = 4) and invasive cancer (5). The sum of scores from three sections from each animal was used as the total dysplasia score per our work and others (12, 17).

Pre-neoplastic changes of the ovary were defined as surface hyperplasia, inclusion cysts, stromal hyperplasia and papilloma, each being a separate histologic parameter (17, 22). For each ovarian section, each parameter was given a score of 0, 1, or 2, based on the severity or prevalence of each pre-neoplastic category (i.e. a score of 0 represented an absence of preneoplastic changes, a score of 1 corresponded to a moderate prevalence or degree of changes and a score of 2 indicated an incidence or high degree of abnormality). Scores for all 4 histologic parameters were added up to give a dysplasia score ranging from 0 to 8 for each ovarian section. These preneoplastic criteria are the same as those used by Stewart *et al.* with this rat model of ovarian carcinogenesis (17).

For both mammary gland and ovarian sections, the number of epithelial cells per high power field was counted manually.

Real Time PCR—The effect of SDG treatments on gene expression in the mammary and ovary of rats (n=6–8/group) were compared using real time PCR. One mammary gland and half of the collected ovary from each rat had RNA isolated using the Trizol reagent and cDNA was generated using high capacity reverse transcription kits (Life Technologies, Grand Island, NY). Real time PCR analysis was conducted using TaqMan Gene Expression Assays with proprietary probes for the mammary genes related to estrogen signaling: Cyp19a1 (Rn01422546), ESR1 and 2 (Rn01640372, Rn00562610), Pgr (Rn01448227) and TFF1 (pS2, Rn00589157); epithelial:mesenchymal transition: CDH1 (Rn00580109), CDH2 (Rn00580099); proliferation: MKI67 (Rn01451446), and housekeeping genes: HPRT1 (Rn01527840), PPIA (Rn00590933), PPIB (Rn03302274), GADPH (Rn01775763), TBP (Rn01455646) and β -Actin (Rn00667869) (Life Technologies, Grand Island, NY). Ovarian tissue was assayed for ESR1 and 2, MKI67, PS2 as well as tumor markers: TP53 (Rn00755717), CA125 (Nbr1, Rn01463598) and WT1 (Rn00580566) with the same housekeeping genes. A reaction mix for each probe was made comprised of 360 μ l TaqMan Master Mix, 36 μ l of respective TaqMan Gene Expression Assay probe, and 252 μ l water. Plates were loaded by first putting 2 μ l of 1:10 dilution of original cDNA per well for each desired cDNA sample, making sure to have duplicates of each sample for each target gene. 18 μ l of the desired target gene's master mix was then pipetted into its designated wells. The plates were run using an ABI PRISM 7000 Sequence Detection System with a cycle

comprising of and initial start of 50 °C for 2 minutes and 95 °C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60 °C for 1 minute. BioGazelle software (GeneNorm, Ghent, Belgium) selected B-actin and PPIA as stably expressed reference genes for data normalization.

Statistical Analysis

Mann-Whitney tests were used to analyze serum lignan and hormone levels; tissue dysplasia scores, epithelial cell numbers, and expression of Ki-67; and gene expression data. Differences were considered significant when $p < 0.05$ (2-sided).

Results

Lignan concentrations in sera

Serum concentrations of secoisolariciresinol parent compound and for total lignans were elevated by SDG administration of 10 ppm ($p=0.020$ and $p=0.043$, respectively, versus controls; Figure 1) Secoisolariciresinol as well as the active metabolites enterodiol and enterolactone, and total lignans were significantly higher in animals that received 100 ppm than in those that received 0 or 10 ($p = 0.028$ for all). Moreover, 100 ppm SDG resulted in serum concentrations of total lignans similar to those seen in our human study (13).

Serum concentrations of ovarian steroids

The continuous estradiol supplementation had the expected effect of raising estradiol concentrations in comparison to the naïve procedural controls ($p=0.008$; Figure 2). There was also a modest effect of decreased progesterone levels when the three carcinogen groups were compared to the controls ($p=0.011$). Compared to the vehicle control, SDG had no effect on serum concentrations of either hormone.

Mammary morphology and histology

Rats receiving E₂/DMBA without SDG supplementation showed dense, enlarged mammary glands on whole mount and multiple layers of ductal epithelial cells on higher magnification (Figure 3). Correspondingly, carcinogen-treatment increased dysplasia scores and mammary epithelial cell number in comparison to naïve controls ($p=0.004$; $p=0.027$, respectively). Ki-67 expression in carcinogen-treated animals was numerically higher than in naïve controls, but this was not statistically significant ($p=0.074$).

Both 10 and 100 ppm SDG were associated with visibly less cellularity with 1–2 layers of ductal epithelia and decreased branching and density on whole in comparison to two or more layers in controls. For all three measures, administration of SDG resulted in median values that were intermediate between those of naïve controls and those of animals treated with carcinogen only (Figure 4). However, only for epithelial cell number was there a statistically significant difference for carcinogen vs 10 ppm SDG ($p=0.046$).

Ovarian histopathology

Local ovarian DMBA application to the ovarian surface epithelium was used to drive epithelial cancer progression. The dysplasia score in carcinogen-treated animals was

increased compared to naïve controls, but was not statistically significant ($p=0.10$). Administration of SDG resulted in median dysplasia values that were intermediate between those of naïve controls and those of animals treated with carcinogen only (Figure 6). Ovarian Ki-67 positive cells were unaltered by treatment. Importantly, administration of SDG did not promote dysplastic progression in the ovary.

Mammary and ovarian cancer biomarker gene expression

Treatment with carcinogen resulted in several changes in gene expression in mammary gland tissue. ESR1 and CDH2 were decreased compared to naïve controls ($P<0.05$ for both); ESR2 was decreased and MKi-67 was increased, but these were not statistically significant. Administration of 1 mg/kg/d SDG reversed these changes, increasing ESR1, ESR2, and CDH2, while decreasing MKi67 when compared to carcinogen alone ($p<0.05$ for all). The higher dose of SDG resulted in similar effect for ESR2 and CDH2 ($p<0.05$) only. For ovarian tissue there was a tendency ($p<0.10$) towards increased CA-125 and decreased ESR1 and TP53 in carcinogen treated animals compared to naïve controls, but only for 1 mg/kg/d SDG was there a suggestion that CA-125 might be decreased ($p<0.10$).

Discussion

This preclinical work emphasizes examination of early changes in tissue-based biomarkers of breast and ovarian cancer risk and progression that directly parallels our active human randomized, placebo-controlled phase II trial of flaxseed lignan, secoisolaricresinol diglucoside (SDG) targeting breast tissue risk biomarkers. The ongoing trial was informed by a previous single-arm pilot study [13] in which 45 healthy pre-menopausal women at high risk for development of breast cancer had benign breast tissue sampled by random periareolar fine needle aspiration (RPFNA) during the follicular phase of the menstrual cycle, both before and after 12-month administration of 50 mg SDG daily. Median serum levels of secoisolaricresinol, enterodiols, and enterolactone were increased from 0.0, 0.0, and 11.1 nmol/L to 5.7, 23.3, and 99.2 nmol/L, respectively. Total lignans increased by a median of 16-fold (11 to 166 nmol/L). Most (80%) of the 45 subjects showed a decrease in Ki-67, from a median of 4% (range, 2–16.8%) to 2% (range, 0–15.2%; $P < 0.001$, Wilcoxon signed rank test). A decrease from baseline in the proportion of women with a cytologic abnormality characterized as hyperplasia with atypia was also observed ($p=0.035$), as were decreases in serum levels of follicular phase SHBG ($p=0.031$) and IGFBP-3 ($p=0.029$). No other statistically significant changes in biomarkers or risk or response were noted.

In the current study, lignans derived from SDG were increased to serum concentrations similar to those seen in human subjects. Specifically, total lignan levels (converted to molarity for ease in comparison) increased from a median level of 11 nmol/L in control animals to 137 nmol/L in animals that received the high dose of SDG. Note that, although SDG and metabolite concentrations were elevated similarly to those seen in human study, the relative abundance of enterolactone vs. enterodiol does differ between human and rats. With attainment of desired lignan concentrations, there was decreased mammary epithelial proliferation and cell number and a suggestion of a decrease in mammary epithelial atypia in a rat model of simultaneous breast and ovarian cancer progression. The proportional

decrease in Ki-67 expression (25% reduction from a median of 5.2% to 3.9%) was reminiscent of the 50% reduction (4.0% to 2.0%) observed in the clinical trial. The mammary dysplasia score decreased from a median of 4 to 2 for rats receiving SDG. Perhaps more comparable is the decrease in the frequency of rats exhibiting a dysplasia score of 4 (the maximum observed) from 63% to 19%. This is consistent with a reduction from 62% to 42% of subjects exhibiting cytologic atypia. Similar to the clinical study, no changes in serum levels of estradiol or progesterone were observed. With these similarities between rat and human responses to SDG, it will be interesting to see whether the changes observed in rats for gene and protein expression will be recapitulated in the ongoing clinical trial.

Consumption of flaxseed in case control studies, as well as comparison of populations with higher vs lower blood levels of lignans, have been associated with reduced breast cancer incidence in human studies (1, 24–26). There is reduced tumor progression and burden when either flaxseed or SDG is supplemented in both xenograft and carcinogen animal models of human breast cancer (2, 27–29). Hypothesized mechanisms of risk benefit include reduction in estrogen related signaling (4, 27, 30), antiangiogenic and antioxidant activity at higher doses for flaxseed (31). Practical advantages of flaxseed or other sources of lignans include minimal side effects, and high subject compliance (13). The results of the current study confirm past preclinical work while strengthening our understanding with an internal dose validation in parallel with human trial of SDG, and test the lignan in an alternative model with E₂-driven mammary carcinogenesis and additional data confirming a lack of ovarian side effects.

The timepoint chosen here (3 month), necessarily focuses on preneoplastic endpoints in both mammary and ovarian tissue, in concordance with our human study. We did see trends for increased P53 expression and decreased ovarian dysplasia in SDG groups in agreement with other groups (6, 32). Most critically, there was no indication that SDG was promoting pre-neoplastic progression in the ovarian epithelium. This is a significant negative finding as some SERMs can cause cystic ovarian disease due to derangement of the hypothalamo-hypophyseal-ovarian axis (7).

Increased ESR2 (ER β) gene expression was the most interesting change in mammary mRNA biomarkers examined. A number of groups have suggested both that 1) ER β signaling can oppose or reverse the proliferative signaling in mammary epithelial tissue (33) and 2) that phytochemicals including SDG might activate this pathway (27, 33). As such, our working hypothesis is that increased ER β signaling is one mechanism behind decreased mammary proliferation and dysplasia seen with SDG administration. Other markers of estrogen signaling (Esr1 and 2, Pgr) were unaltered by SDG at any dose. N-cadherin (CDN2) was elevated in SDG-treated mammary glands as well. While elevated N-cadherin is associated with an epithelial to mesenchymal transition in cancer metastasis, a more likely explanation here is altered composition of normal mammary tissue due to decreased epithelial cell numbers with SDG administration, particularly an increased relative abundance of vascular or stromal tissue and decreased epithelial component which enriches for Cdh2 expression (34).

In summary, treatment with SDG at doses mimicking those used in human trial resulted in comparable concentrations of SDG and its metabolites in sera. This, in turn, reversed the proliferative and dysplastic effects of estrogen on mammary tissue. SDG did not promote ovarian dysplasia in this model.

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Figure 1.

Serum concentrations of secoisolariciresinol (A), its major metabolites enterodiols (B) and enterolactone (C), and total lignans (D) after 3 weeks of administration of vehicle control, low dose SGD (10 ppm in feed) or high dose SGD (100 ppm, n=4 in each group). SGD administration elevated concentrations of secoisolariciresinol, enterodiols, enterolactone, and total lignans in a dose dependent fashion.

Figure 2.

Serum concentrations of 17β-estradiol (A) and progesterone (B) at 3 months after initiation of carcinogen and treatment with vehicle control, 10 ppm SGD or 100 ppm SGD (n=6 each) in feed. All carcinogen and SGD groups received sustained release estradiol treatment. Median and full range values are shown; the dashed lines denote median hormone concentrations in the procedural controls. While the carcinogen treatment elevated estradiol and reduced progesterone, administration of SGD had no effect on concentrations of these ovarian steroids.

Figure 3. Histology (Carmine, 5×) of whole mount mammary tissue obtained at 3 months from a procedural naïve control (no carcinogen) rat, a rat treated with carcinogen (sustained release E₂/ovarian DMBA) and vehicle control, and carcinogen-treated rats that received 10 or 100 ppm SDG. Rats receiving carcinogen alone had confluent mammary epithelia obscuring the ductal tree. Both SDG groups had fewer epithelia, intermediate to carcinogen controls and naïve, age-matched controls.

Figure 4.

Effects of SGD on epithelial dysplasia (A), epithelial cell number (B) and Ki-67 positive cells (C) in mammary glands after 3 months of carcinogen with and without SGD treatment (n=8–10/group). Median and full range values are shown; the dashed lines denote median hormone concentrations in the procedural controls. The carcinogen treatment resulted in increases in dysplasia score, epithelial cell number, and a trend towards more Ki-67 positive cells. Administration of 10 ppm SGD normalized the epithelial cell number.

Figure 5.
Effect of SGD on ovarian epithelial dysplasia after 3 months of carcinogen with and without SGD treatment (n=8–10/group). Median and full range values are shown; the dashed lines denote median hormone concentrations in the procedural controls.