Influence of Prevastein®, an Isoflavone-Rich Soy Product, on Mammary Gland Development and Tumorigenesis in Tg.NK (MMTV/c-neu) Mice

Anni R. Thomsen, Alicja Mortensen, Vibeke M. Breinholt, Rikke H. Lindecrona, José L. Peñalvo, and Ilona K. Sørensen

Abstract: We investigated spontaneous mammary tumor development and mammary gland morphogenesis in female Tg.NK mice postnatally exposed to dietary soy isoflavones (0, 11, 39, and 130 mg aglycones/kg diet) added to a Western-style diet. Instead of preventing mammary tumorigenesis, the highest dose of isoflavones was associated with a small but significant increase in the number and size of tumors as compared to mice administered a Western-style control diet (P < 0.05). At postnatal Week 6, dynamic activity (measured as apoptotic density) at the highest dose and the degree of branching of the mammary tree in all isoflavone-exposed groups was increased as compared to controls (P < 0.05). At adulthood, the epithelium appeared more quiescent in the medium- and high-dose groups evident by reduced apoptotic density and a reduction in the percentage of terminal end buds (TEBs), respectively, as compared to controls (P < 0.05). The number of actively dividing cells within the TEBs was unaffected by isoflavone exposure as was the activity of drug-metabolizing and antioxidant enzymes. In conclusion, isoflavones may augment mammary gland and mammary tumor development.

Introduction

Soy isoflavones, nonsteroidal diphenolic compounds that are especially abundant in the soybean, are extensively used in dietary supplements as cancer preventives and alleviators of postmenopausal symptoms. The results on the impact of isoflavones on mammary tumor formation is, however, controversial. Epidemiological studies have shown that incidence and mortality of breast cancer is fivefold lower in Asian countries as compared to Westernized countries (1). This difference in disease incidence is often attributed to increased consumption of soy-containing products by Asian women (2–6). The observation that Asian female immigrants in the United States have a significantly lower risk of acquiring mammary cancer, whereas this protection is not evident in the second generation, indicates the importance of diet composition in the etiology of mammary carcinogenesis (7). Soy isoflavones have accordingly been extensively studied for their potential ability to exert cancer preventive effects in humans. The predominant isoflavones in soy are genistein and daidzein. Genistein and daidzein activate estrogen receptors alpha (ERα) and beta (ERβ) and induce an estrogenic response in both in vitro and in vivo studies (8–11). Genistein has additionally been found to inhibit proliferation of both ERα-positive and ER-negative human breast and prostate cancer cell lines at physiological doses (12) but to stimulate the growth of ERα-positive breast cancer cells at pharmacological doses (13). Genistein also acts as a specific and potent inhibitor of protein tyrosine kinases and topoisomerase II (14,15), and inhibits angiogenesis and metastasis in several tumor models (16,17). Recent evidence suggests that lifelong consumption of phytoestrogens, particularly soy isoflavones, may reduce breast cancer risk (18) and that exposure during childhood and adolescence may be important in providing a persistent protection against breast cancer (19,20). Animal studies have provided evidence that administration of genistein modulates chemical carcinogenesis in rodents and that the impact of genistein on carcinogenesis seems to depend on the specific time interval of soy exposure (21–28). It is suggested that prepubertal exposure to genistein alters the differentiation of the mammary epithelium (21,29). The terminal end bud (TEB) constitutes the invading front of the mammary epithelium and is a potential site for malignant transformation due to its undifferentiated nature. Induced differentiation of the mammary gland resulting in fewer TEBs may explain the cancer protective effect of genistein in rodent carcinogenesis (30). Here, we evaluate the effect of an isoflavone concentrate from a natural whole-soy product, Prevastein®, on mammary gland morphogenesis and tumor formation in the Tg.NK (mouse mammary tumor virus

A. R. Thomsen, A. Mortensen, R. H. Lindecrona, and I. K. Sørensen are affiliated with the Danish Institute for Food and Veterinary Research, Department of Toxicology and Risk Assessment, DK–2860 Søborg, Denmark. V. M. Breinholt is affiliated with Maxygen Aps, DK–2970, Hørsholm, Denmark. J. L. Peñalvo is affiliated with the Institute for Preventive Medicine, Nutrition and Cancer, Folkhålsans Research Center, and Division of Clinical Chemistry, 00014–University of Helsinki, Finland.
[MMTV]/c-neu) mice. This animal model carries an activated c-neu oncogene, a homologue to human ErbB-2/HER-2, containing a mutation in the transmembrane region of the receptor under the control of the MMTV long terminal repeat. The subsequent uniform expression of the activated MMTV/c-neu oncogene results in the development of independent but multiple mammary tumors between 5 and 10 mo of age. Tumors arise adjacent to morphologically normal epithelium and metastasize but do not develop other histological types of tumors in other tissues by the 7th mo of age (31). The latency period of approximately 18 wk from weaning until development of palpable mammary tumors makes this model suitable for diet intervention studies. Tg.NK mice were fed a Western-style diet (high fat, low calcium) with or without Prevastein added from weaning until termination of the study. In addition, the influence of Prevastein on the rate of apoptosis in the developing mammary gland, the number of actively dividing cells within the different epithelial structures, the activity of selected drug-metabolizing enzymes, and the activity of a marker for redox status in red blood cells (RBCs) was studied in this animal model.

Material and Methods

Test Compound and Diets

Prevastein, a soy isoflavone concentrate, was obtained from Central Soy company, Inc., Port Wayne, Indiana. According to the manufacturer, the batch used in this study contained 46.19% (wt/wt) of isoflavones of which 66.5% was genistein, 32.3% daidzein, and 1.2% glycinechin. The other constituents in Prevastein were carbohydrates (29%), protein (17.6%), crude fiber (0.5%) and fat (<0.1%). Four types of pelleted Western-style diets high in animal fat (20% wt/wt; equal to approximately 40% energy), moderate in fiber (9% wt/wt), and low in calcium (3 g/kg diet) were prepared by the Special Diet Service, United Kingdom: A control diet and this diet added 0.004% (wt/wt), 0.02%, or 0.06% of Prevastein (Table 1). These diets were formulated as described previously in the framework of the European Union (EU)-funded project EU-FAIR (32,33). The fiber-rich, nonpurified, NTP–2000 diet was provided by Ziegler Bros Inc., Gardners, PA. Detailed information on ingredients and nutrients in the NTP–2000 diet and the tumor development in Tg.NK mice kept on this diet has been published elsewhere (34). Altromin 1324 was provided by Altromin GmbH u., Co KG, Lage, Germany.

Animals, Housing, and Clinical Observations

A total of 160 female hemizygous Tg.NK mice (offspring of homozygous Tg.NK male transgene carriers bred to FVB/N wild type female mice) of approximately 3 wk of age and weighing 12.6 g ± 1.6 (mean ± SD) were obtained from Taconic Farms, Inc., Germantown, NY. All female Tg.NK mice were housed as 3–5 per cage. To prevent the occurrence of anoestrus, four male NMRI mice approximately 4 wk old were placed in the same room as Tg.NK female mice. The males were obtained from Bommice Bonholtgaard Breeding and Research Center LTD, Rye, Denmark, housed one per cage, and fed Altromin 1324 diet. All mice were kept under controlled environmental conditions (temperature 21 ± 1°C, the relative humidity 55 ± 5%, 12/12 h light/dark cycle, air changed 10 times/h) and had free access to food and water acidified to pH 3.0 by citric acid (to prevent growth of microorganisms). Body weight and feed intake were recorded once weekly for female Tg.NK mice only. All mice were observed at least twice a day for any abnormalities in clinical appearance.

Experimental Design

Tumor study: The female Tg.NK mice were allocated to five groups based on body weight and fed the experimental diets from the 25th day of life for 24 consecutive weeks: Group 1 of 22 mice a Western-style control diet (no Prevastein) and Group 2 of 20 mice and Groups 3 and 4 of 21 mice each a Western-style diet added with 0.004%, 0.02%, or 0.06% of Prevastein, respectively. Group 5 of 21 mice serving as a model control received NTP–2000 diet.

Starting at the 20th wk of life, the animals were palpated for the presence of subcutaneous tumors twice a week. All mice that became moribund or with markedly disturbed general conditions during the study were anaesthetized by CO2/O2 inhalation, bled to death, and autopsied. At termination, all the remaining Tg.NK mice were anaesthetized by an intraperitoneal injection of a pentobarbital solution (60 mg/kg body weight). From an uncovered thoracic cavity, blood samples were collected by heart puncture and stabilized in heparin. The ventral skin was separated from the peritoneum, and all visible subcutaneous tumor masses were counted, measured with calipers, removed, weighed (those

<table>
<thead>
<tr>
<th>Component</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn starch</td>
<td>210</td>
</tr>
<tr>
<td>Sucrose</td>
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</tr>
<tr>
<td>Casein</td>
<td>230</td>
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<td>Lard</td>
<td>147</td>
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<td>60</td>
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</tr>
<tr>
<td>Methionine (synthetic)</td>
<td>3</td>
</tr>
<tr>
<td>Total (g)</td>
<td>1,000</td>
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</tbody>
</table>

b: K 8408.07 RIVM.
c: Low calcium K8418.07 RIVM.
with diameter below 2 mm were not weighed), and fixed in 4% neutral buffered formaldehyde. The thoracic and abdominal cavities were examined for gross lesions. Liver and uterus were removed and weighed. All the subcutaneous tumor masses were processed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for light microscopic examination.

Tumor burden was reported as the number of and weight of tumors per mouse.

**Interim sacrifices for studies of mammary gland development:** A total of 54 female Tg.NK mice were used for investigation of mammary gland development. Six untreated mice were sacrificed just prior to experimental feeding. The other mice were divided into four groups (5–6 animals per group) and fed specific Western-style diets as Groups 1 to 4 in the main study. The mice in estrus, as determined by vaginal smear (35), were anesthetized, and blood samples were collected as described previously. Immediately thereafter, the arterial system was washed with 10 ml ice cold sterile phosphate-buffered saline through the cannula inserted to the left ventricle. The ventral skin was reflected, and mammary tissue from Glands 3, 4, and 5 was collected as described in the respective sections following; the liver and uterus were removed, weighed, frozen in liquid nitrogen, and stored at –80°C until biochemical analysis. Animal studies and all procedures were approved by the Danish Animal Experimental Inspectorate.

**Isoflavone concentrations in diets:** The levels of daidzein, genistein, and glycitein in the diets of the animals were quantified by high-performance liquid chromatography with coulometric electrode array detection as previously described (36,37). Briefly, approximately 50 mg of the sample was weighed, and 2.5 mL of 80% ethanol (EtOH) was added. Samples were vigorously shaken for 2 min and centrifuged 2,140 g. Supernatant was decanted into 10 mL volumetric flask and extracted twice applying the same procedure. Chromatographic system (ESA Inc., Chelmsford, MA) was set up in accordance with the previously described protocol: isoflavones were separated using an Inertsil ODS-3 (GL Sciences Inc., Tokyo, Japan) column (150 × 3 mm, 3 μm) protected with a Quick Release C18 (Upchurch Scientific Inc., Oak Harbor, WA) column (10 × 3 mm, 5 μm). The mobile phase consisted of two eluents: 1) 50 mM sodium acetate buffer pH5/methanol (MeOH; 80:20 vol/vol) and 2) 50 mM sodium acetate buffer pH5/MeOH/acetonitril (ACN; 40:20:20 vol/vol/vol) applied in gradient. Injection volume was 10 mL. Detector consisted of eight electrode pairs set between 200 mV and 700 mV, and quantification was made by external calibration with authentic reference standards.

**Isoflavone Concentrations in Plasma**

Heparinized blood samples were centrifuged at 1,500 g for 10 min at 4°C to separate plasma from RBCs. Concentrations of daidzein and genistein concentrations in plasma were quantified by time-resolved fluoroimmunoassay (TR-FIA) accordingly to a previously described protocol (38). In summary, 200 μL of plasma was first added with 20 μL of 3H-estradiol glucuronide to assess recovery during the sample pretreatment. After 30 min at room temperature, 200 μL of a hydrolytic reagent containing sulphatase (2 U/mL) and β-glucuronidase (0.2 U/mL) were added, and the samples were incubated overnight at 37°C to release the isoflavone aglycones. Extraction was performed twice in Vortex using diethyl ether. After separation by freezing, the organic phases were collected and evaporated to dryness. A volume of 200 μL of 0.5% bovine serum albumine (BSA) Tris-buffer pH 7.8 was then used to reconstitute the extracts by thorough mixing. An aliquot of 20 μL was transferred to a liquid scintillation counter to determine the recovery percentage, and two additional aliquots of 20 μL each were taken to perform TR-FIA of daidzein and genistein. Aliquots were placed into goat antirabbit IgG microtiterplate strips and 100 μL antiserum in BSA Tris-buffer was added together with 100 μL of the europium-labeled daidzein and genistein. After incubation and shaking for 90 min at room temperature, the strips were washed, and 200 μL of enhancement solution was added to each well to develop fluorescence that was subsequently measured in a VICTOR 1420 (Wallac Oy, Turku, Finland) multilabel counter.

**Mammary Whole Mount Preparation and Morphometric Analysis**

Mammary whole mounts were prepared from the fourth abdominal gland on the right side from five to six mice per group. Glands were spread on SuperFrost® Plus microscope slides (Menzel-Glaser, Braunschweig, Germany) fixed in ethanol: acetic acid (3:1 vol/vol) for 2 h, rehydrated in 70% ethanol (15 min), rinsed in water (5 min), and stained with carmine alum (2 g/L) overnight (14–18 h) at room temperature. The stained glands were dehydrated in grading ethanol (70–100%) in three steps (15 min/step), cleared in xylene for several days, and permanently mounted with Permount and a coverslip. The mammary gland of each mouse was evaluated by examining the whole mounts under a light microscope equipped with ocular micrometer. The length of a straight line from the lymph node to the end of the most distal terminal branches represented the relative length of the mammary tree. Although branching morphogenesis occurs by two different mechanisms—branching by end bud bifurcation and by lateral sprouting of mature ducts to form secondary branches (39)—we did not discriminate between the two. Branching was measured as the average of the mean number of branch points along three of the longest primary ducts relative to the actual length of those ducts. The stage of gland differentiation was evaluated by the occurrence of TEBs, terminal ducts (TDs), alveolar buds (ABs), and lobular structures in the area most distal to the lymph node based on the classification by Russo and Russo (40). The TEB was recognized as a characteristic bulbous structure more than 100 μm in diameter. The TD appeared as a blunt-tipped structure less
than 100 μm, whereas the AB was identified as a terminal or a lateral bud that had differentiated by further sprouting into 3 to 10 smaller buds. The lobule was recognized by its grape-like structure consisting of ABs.

**Immunohistochemical Examination of Proliferative Cell Nuclear Antibody (PCNA) Levels**

The fifth mammary gland was removed and fixed in 4% natural buffered formalin for 24 h before it was processed and embedded in paraffin. Four serial sections of 3.5 μm with 35 μm interval from the second abdominal mammary gland from each mouse were dried overnight at 37°C, deparaffinized in petroleum, and rehydrated in grading ethanol (99%, 96%, 70% EtOH and tap water). Epitope retrieval was performed by heat treatment 2 × 5 min in a microwave oven at 100°C in 10 mM sodium citrate, pH 6. Unspecific binding was blocked with 2% BSA. The primary antibody, anti-PCNA (DAKO M0879), was applied with DAKOs Animal Research Kit (mouse). TEBs and ABs were recognized by their morphology and scored blindly by two investigators using the following criteria: 0 = 0–10%; 1 = 10–25%; 2 = 25–50%; 3 = 50–75%; and 4 = 75–100% positive cells per end bud or duct.

**Apoptosis Analysis**

The thoracic mammary glands from each mouse were frozen immediately after removal in liquid nitrogen and stored at −80°C until use. DNA was isolated as published elsewhere (41). Detection of nucleosomal ladders was based on ligation-mediated polymerase chain reaction (LM-PCR) using the ApoAlert LM PCR ladder Assay Kit Number K905–1 from Clontech (43). LM-PCR was designed specifically for the detection of nucleosomal ladders in apoptotic cells and to enable visualization of DNA ladders that were undetectable by other methods. The LM-PCR assay was semiquantitative, allowing comparison of the relative extent of apoptosis in different samples.

The densitometric analysis of the apoptotic banding pattern visible on the agarose ethydium bromide stained gel was accomplished using Alpha Ease FCTM Stand Alone Software for Windows 95/98 NT2000 catalog IA–800–98 (AlphaInnotech Corporation, San Leandro, CA). Using this densitometer software, outlines with equivalent areas were drawn around the bands, and the intensity of the total area was determined.

**Determination of Drug-Metabolizing and Antioxidant Enzyme Activities**

Whole liver from each mouse was homogenized in 3 volumes of buffer [0.05 M 3-morpholinopropansulfonic acid (MOPS), 1.0 M ethylene-diaminetetra-acetic acid, pH 7.4], Cytosol and microsomes were separated as described elsewhere (42) and stored in MOPS buffer containing 20% glycerol at −80°C until use. Protein determination was done with Pierce Bicinchoninic Acid Protein Assay Reagent kit (Rockford, IL). The cytochrome P450 (CYP450) activity profile in the microsomes was determined by use of alkyl-resorufin derivatives, which are substrates for various CYP450 enzymes (45, 46). In brief, ethoxyresorufin-O-deethylase (EROD) activity was measured in duplicates on liver microsomes in 96-well plates (Black Optiptate, Packard, Randburg, SA). The reaction was performed with 6 μg liver microsomal protein in 0.1 M Tris-hydrochloric acid (pH 7.8) containing 2 μM ethoxyresorufin, 1.6 mg/ml BSA in a total volume of 0.2 ml. The mixture was preincubated at 37°C for 2 min, and the reaction was started by the addition of 0.25 mM nicotinamide adenine dinucleotide phosphate. Resorufin-associated fluorescence was measured at 560 nm excitation and 590 nm emissions using a fluorescence reader (Victor2, Wallac, Perkin Elmer Life Sciences, Boston, MA). The reaction was followed for 30 min with readings every 4 min, and the rate of resorufin production was calculated. Benzyloxy- (BROD), methoxy- (MROD), and pentoxyresorufin-O-deethylase (PROD) activity was measured following the same procedure as described previously for EROD, with final concentrations of 2 μM substrate (PROD, BROD, or MROD) and 6 μg microsomal protein per well.

The activities of quinone reductase (QR) and glutathione S-transferase (GST) in liver cytosol were determined according to the methods described elsewhere (47, 48), respectively, adapted to a Cobas Mira (GMI, Inc., Ramsey, MN) analyzer. GST activity was determined as μmol/min/g protein of conjugate with 1-chloro-2,4-dinitrobenzene formed and QR as μmol cytochrome c reduced/min/g protein. The samples were diluted 300 times prior to analysis. All samples were analyzed in duplicate.

Measurement of the glutathion peroxidase (GPx) activity in RBCs was performed as described elsewhere (49). In brief, heparinized blood samples were centrifuged at 1,500 g for 10 min at 4°C to separate plasma from RBCs. The RBC fraction was resuspended in 1 volume of deionized water and stored at −80°C. Automated assays were performed with a Cobas Mira S analyzer, and commercially available kits were used for determination of GPx activity and hemoglobin concentrations. The enzymatic activity was calculated relative to the amount of hemoglobin measured. Samples were analyzed in duplicates.

**Statistical Analysis**

Data was presented as means ± SD where appropriate. Before subjected to further analysis, all data were tested for normal distribution by Shapiro–Wilks test, and the homogeneity of variance among the groups was evaluated by judgment of standardized residuals plot. As the data on body weight and feed intake showed the homogeneity of variance and were normally distributed, it was analyzed by Dunnett’s test (Group 1 vs. 4) or by Student’s t-test (Group 1 vs. Group 5). Data on survival was analyzed using the lifetest procedure.
The data on number, weight, and size of tumors per tumor-bearing mouse at necropsy was analyzed by analysis of variance, followed by Student's t-test for the means (unpaired, two-tailed) for pairwise comparison. The same was performed on data of mammary gland morphology and apoptotic density. Values of \( P < 0.05 \) were considered statistically significant. All statistical analyses were performed using Statistical Analysis System (SAS) software (Version 6.12; SAS Institute Inc., Cary, NC).

**Results**

**Isoflavone Content in Diets and Plasma**

The analysis of isoflavone content in the diets (Table 2) confirmed their presence at increasing levels according to the three different concentrations of Prevastein. Subsequently, the measurement of plasma concentrations of daidzein and genistein confirmed their absorption. Their increased levels in all Prevastein exposed mice and of mice on NTP-2000 diet demonstrated an enhanced systemic exposure to soy isoflavones of the mice in all test groups compared to the group on the control diet.

**Effects on Clinical Appearance, Body Weights, Survival, and Organ Weights**

The clinical appearance of mice did not differ among the various exposure groups. With regard to behavior, the Tg.NK mice appeared very active and tended periodically to run in circles. Body weights of mice on diets added with a low, medium, and high dose of Prevastein were comparable to those of mice fed a control diet from the start of the experimental feeding until the termination (Fig.1). The body weight of mice fed the NTP-2000 diet was significantly lower \( (P < 0.05) \) compared to that of mice on the Western-style diet from the 2nd wk of treatment until termination, with exceptions at the start and in Weeks 3, 7, 18, 19, and 21. At the time of necropsy, the contribution of tumors to the overall body weight was below 1.26% in all diet groups. The only statistically significant difference recorded in relative feed intake compared to the control diet was a decrease for the group receiving high concentration of Prevastein. Mammary tumors of Tg.NK mice were of glandular epithelial origin with a pleomorphic growth pattern and were classified as adenocarcinomas type B. No difference in histological appearance of mammary tumors was seen between the groups.

**Effects on Mammary Tumorigenesis**

The pattern of mammary tumor incidence from the postnatal Week 21 to 27 is shown in Fig. 2. Palpable tumors were observed from postnatal Week 23 in all Western-style diet groups. By postnatal Week 27, approximately 60% of the mice on the Western-style diets had tumors. The majority of tumors were located in the thoracic mammary glands. The study was terminated through postnatal Week 28. The incidence of mammary tumors at necropsy reached 90–100% in all groups (Table 3). The tumor incidence and time to tumor in the NTP-2000 diet group, which served as a control diet for the animal model, was consistent with that previously reported (34).

A significant increase in the average number of tumors per tumor-bearing mouse was found in the group receiving the highest concentration of isoflavones as compared to the control group \( (P < 0.05) \). In addition, the average size of tumors was significantly increased in animals receiving high concentration of Prevastein. Mammary tumors of Tg.NK mice were of glandular epithelial origin with a pleomorphic growth pattern and were classified as adenocarcinomas type B. No difference in histological appearance of mammary tumors was seen between the groups.

**Effects on Mammary Gland Morphology**

Whole mounts of mammary glands from Tg.NK mice at postnatal Week 6 and 10 are presented in Fig. 3. At postnatal Week 6, TEBs (Fig. 4A) constituted 50–60% of all end structures of the ducts but some regression into TDs and further differentiation into ABs is seen. By postnatal Week 10, the number of TEBs was diminished further, whereas the amount of ABs was increased but varied considerably between individual animals (Fig. 4B). The mammary gland morphology of Tg.NK mice was found to be comparable to the morphology of FVB wild type mice (data not shown). Morphometric analysis of the mammary epithelium at postnatal Week 6 showed no statistically significant difference in the relative length of the mammary tree in the Prevastein fed groups compared to the controls, but a significant \( (P < 0.05) \) increase in branching of ducts in all Prevastein fed groups was found at postnatal Week 6 (Fig. 4). At postnatal Week 10, the morphometry and length of the mammary tree was similar in all groups. When comparing epithelial structures, no difference was observed among the groups at postnatal Week 6 (data not shown), but at postnatal Week 10, a significant reduction \( (P < 0.05) \) in the number of TEBs was observed in the medium-dose group (Fig. 5). No difference in the number of alveolar buds or lobules was found (data not shown).
Table 2. The Measured Content of Isoflavones in Western-Style Diets With Added Prevastein® and in NTP-2000 Diet, Mean Relative Feed and Isoflavone Intakes, and Plasma Concentration of Daidzein and Genistein in Tg.NK Mice\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose of Isoflavones</th>
<th>Isoflavone Content in the Diet (Mg/g)</th>
<th>Relative Feed Intake (g/kg bw/day)</th>
<th>Isoflavone Intake\textsuperscript{d} (mg/kg bw/day)</th>
<th>Isoflavone Concentration in Plasma (nM)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Daidzein</td>
<td>Genistein</td>
<td>Glycitein</td>
<td>Total</td>
</tr>
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<tr>
<td>2</td>
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<td>4</td>
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<td>46.31</td>
<td>80.64</td>
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</tr>
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</table>

\textsuperscript{a}: Abbreviation is as follows: bw, body weight.
\textsuperscript{b}: Statistical significance is as follows: *, \( P < 0.05 \) compared to Group 1, Dunnett’s test.
\textsuperscript{c}: As aglycones.
\textsuperscript{d}: Calculated based on relative feed intake and total isoflavone content in the diet.
Effects on Proliferative Activity in Mammary Epithelial Structures

Semiquantitative evaluation of serial sections of PCNA-stained mammary epithelium at postnatal Week 6 and 10 showed that the number of proliferative cells within the individual TEB was comparable in all groups (data not shown).

Effects on Apoptosis Within the Mammary Gland

The relative level of apoptosis in the mammary gland within each group was significantly higher at postnatal Week 6 than in postnatal Week 10 ($P < 0.001$). Furthermore, the relative apoptotic density at postnatal Week 6 was increased in the high-Prevastein group as compared to the control group ($P < 0.05$). In contrast, the relative apoptotic density in glands at postnatal Week 10 was decreased significantly in the high-Prevastein group as compared to the control group ($P < 0.05$; Fig. 6).

Enzyme Activities

The CYP450 activity in hepatic microsomes was similar in all Western-style diet groups (with or without Prevastein) at postnatal Week 6 and 10 (Table 4). Likewise, the activity of the drug-metabolizing enzymes, GST and QR, in liver cytosol and the antioxidant enzyme, GPx, in RBCs was similar in all Western-style diet groups at postnatal Week 6 and 10 (Fig. 7).

Discussion

Results of this study indicate that postnatal exposure to dietary soy isoflavones, resulting in plasma levels comparable to those in human populations consuming a soy-rich diet (50), stimulates mammary gland morphometry during puberty, reduces the proliferative activity at adulthood, and yet seems to increase spontaneous tumor development in female Tg.NK mice. As 20–40% of all human mammary tumors are found to overexpress ErbB-2 (51), the Tg.NK animal model seems relevant for studying in vivo dietary effects on the development of c-neu/ErbB-2/HER-2 positive tumors. The test diet was a high-risk, Western-style diet designed to simulate the nutrient composition in the Western part of the world. The group receiving a nonpurified diet, NTP-2000, with a lower fat content (20% wt/wt vs. 8.8 % wt/wt) served as a control diet for the animal model (34).

Numerous animal studies of carcinogen-activated tumorigenesis have been performed in rodents with genistein as the prime isoflavone of choice. These studies show that administration of genistein neonatally or perinatally inhibits dimethylbenzoic acid (DMBA)-induced tumorigenesis (21,22,52,53), whereas for mice fed genistein postweaning, an increase in DMBA-induced mammary adenocarcinomas is observed (28). One of the chemopreventive mechanisms of genistein could be its ability to influence mammary gland development and modulate the number of TEBs (21,23,24,54). Most likely, when genistein is given neonatally, it initially increases the proliferation of the mammary epithelium through activation of the epidermal growth factor pathway, which leads to a more mature epithelium in the adult virgin mouse characterized by a diminished number of TEBs (21,23,24,54). Although this study does not support a cancer preventive effect of soy isoflavones, investigations of the mammary gland indicated an early growth stimulatory effect of soy isoflavones. First, at postnatal Week 6, branching of the mammary gland in all groups receiving isoflavones was increased significantly ($P < 0.05$) as compared to the control group. However, this effect on branching morphogenesis seemed transient, as branching in isoflavone-treated groups was comparable to the control group at postnatal Week 10. Secondly, when TEBs proliferate and invade the fat pad during ductal morphogenesis, apoptosis occurs simultaneously in the surrounding stroma and within the TEBs, forming the lumen (56). Because a general age-dependent decrease in the apoptotic...
Table 3. Relative Weight of Chosen Organs, Number, Weight, and Size of Tumors<sup>a,b</sup>

<table>
<thead>
<tr>
<th>Dose of Isoflavones</th>
<th>n</th>
<th>Liver</th>
<th>Uterus</th>
<th>Ovaries</th>
<th>Tumor Incidence (%)</th>
<th>Tumor Multiplicity&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Tumor Weight (g)/mouse&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Tumor Size (mm&lt;sup&gt;2&lt;/sup&gt;)&lt;sup&gt;f&lt;/sup&gt;</th>
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<tr>
<td>Control</td>
<td>22</td>
<td>42.24 ± 4.60</td>
<td>3.51 ± 1.32</td>
<td>0.64 ± 0.16</td>
<td>91%</td>
<td>5.95 ± 2.93</td>
<td>0.40 ± 0.44</td>
<td>1.63 ± 0.71</td>
</tr>
<tr>
<td>Low</td>
<td>16</td>
<td>42.12 ± 6.27</td>
<td>2.99 ± 0.68</td>
<td>0.60 ± 0.19</td>
<td>100%</td>
<td>6.50 ± 4.56</td>
<td>0.47 ± 0.60</td>
<td>1.79 ± 0.87</td>
</tr>
<tr>
<td>Medium</td>
<td>20</td>
<td>41.15 ± 6.21</td>
<td>3.34 ± 1.35</td>
<td>0.64 ± 0.12</td>
<td>95%</td>
<td>7.11 ± 3.73</td>
<td>0.50 ± 0.60</td>
<td>2.23 ± 1.93</td>
</tr>
<tr>
<td>High</td>
<td>18</td>
<td>44.74 ± 5.17</td>
<td>2.91 ± 1.09</td>
<td>0.59 ± 0.21</td>
<td>100%</td>
<td>7.79 ± 2.59*</td>
<td>0.52 ± 0.74</td>
<td>3.04 ± 1.71*</td>
</tr>
</tbody>
</table>

<sup>a</sup>: Data are presented as the mean ± SD when appropriate. n = number of animals per group.
<sup>b</sup>: Statistical significance is as follows: *, P < 0.05 compared to Group 1, Student’s t-test (log transformed).
<sup>c</sup>: Average of the relative organ weight [relative organ weight = 100 × (weight of organ/body weight)].
<sup>d</sup>: Average number of tumors per tumor-bearing mouse ± SD.
<sup>e</sup>: Average tumor weight per tumor-bearing mouse ± SD.
<sup>f</sup>: Average size of tumors.
Figure 2. Incidence of palpable mammary tumors in all groups from the 21st to the 27th wk of life.

Figure 3. Whole mounts prepared from the fourth abdominal mammary gland from Tg.NK virgins. A: At postnatal Week 6. B: Postnatal Week 10. In both glands, terminal end buds (TEBs; arrow heads) are seen at the invading front most distal to the nipple, whereas alveolar buds (ABs) are seen more proximal to the nipple. Regression to terminal ducts (TDs) is seen (small arrow). In both glands, lateral side branching is seen (white arrow). C: Photograph of an alveolar structure from B was taken through a microscope under ×25 magnification.

Figure 4. Analysis of epithelial morphogenesis at postnatal Week 6. A: Mean length of the three longest ducts relative to the lymph node. B: Mean number of bifurcative and lateral branches per length of duct averaged over the three longest ducts of the gland. The bars represent the mean ± SD of glands from six animals. The branching morphogenesis was significantly increased in all treated groups compared to controls (two-tailed, unpaired, Student’s t-test).
density between samples from 6- and 10-wk-old animals was recorded in this study, it seems reasonable to assume that the level of apoptotic density reflects the proliferative dynamic activity within the gland. Further analysis of the level of apoptosis between groups on the control diet and Prevastein-added diets suggested that isoflavones may exert a stimulatory effect on the dynamic activity within the gland at postnatal Week 6, rendering it less active at postnatal Week 10. Also, it seemed that soy isoflavones reduced the percentage of the highly proliferative TEB at postnatal Week 10, as a significant reduction was found in the medium-dose group compared to controls ($P < 0.05$). A reduction in the percentage of TEBs was also observed in the high-dose group, although not statistically significant ($P < 0.06$).

Thus, our findings indicate that dietary soy isoflavones stimulate juvenile ductal growth resulting in a more quiescent epithelium at adulthood in accordance with the suggestion mentioned previously (21,27,55). Because we found that the number of actively dividing cells within the individual TEBs at postnatal Week 6 and 10 was unaffected by isoflavone exposure, it seems that isoflavonoids do not contribute to progression of mammary tumors by increasing local population of cells that are susceptible to tumor formation.

Most studies of rodent tumorigenesis in response to soy isoflavones have been performed with carcinogen-activated tumorigenesis models. The major difference between these studies and this study is the time of initiation of the tumor process. In the studies of carcinogen-activated tumorigenesis, the carcinogen has been administered in late adolescence (after postnatal Week 7). At this age, the mammary gland of genistein treated animals is believed to have become more mature and thus more protected against carcinogenesis (21,27). By contrast, in this study, the expression of the activated oncogene was initiated at onset of puberty at postnatal Week 4. Consequently, the tumor transformation may occur within a narrow scope of increased proliferative activity in the gland that may be induced by soy isoflavones, and therefore, these compounds could accelerate tumor development. Hence, whether to expect protection against or increased risk
of breast cancer on isoflavone exposure in this model may depend on the timing of exposure relative to tumor initiation. The latter could explain the significantly ($P < 0.05$) increased number of tumors per mouse observed in this study in the group receiving the highest amount of Prevastein as compared to the control.

Both genistein and daidzein have a protective effect in form of increased latency period when administered to MMTV/c-neu mice from postnatal Week 7 (57). However, this strain expresses the wild-type form of the receptor; thus, tumorigenesis is not comparable to the strain used in this study. As the Western-style diet alone decreased the latency period by several weeks and increased the tumor incidence, the possible protective effect of isoflavones could be masked by the (harmful) effect of the Western-style diet herein. Nonetheless, it seems that the cancer-promoting effect of the highest dose of Prevastein observed in our study was caused by early puberty exposure, and might be explained by induced proliferative activity of the mammary gland. This is in agreement with another study in which perinatal exposure of Tg.NK (MMTV/c-neu) mice to soy isoflavones resulted in a similar increase in tumor incidence (58). Thus, puberty and early puberty seem to be critical periods to isoflavone exposure with regard to increased breast cancer risk in the adult mouse.

As dietary genistein is known to stimulate the growth of transplanted ER-positive cancer cells in vivo, another explanation for the observed increase in tumor incidence herein could be that soy isoflavones promoted the growth of existing tumor cells directly (59,60). This supposition is supported by the increased tumor size observed for Tg.NK mice treated with a high dose of Prevastein compared to controls ($P < 0.05$). Immunohistochemical staining for estrogen receptors showed that the investigated tumors were positive for ER-$\alpha$ but not ER- $\beta$ (data not shown).

The reduced latency, increased tumor incidence, and increased number of tumors per tumor-bearing mouse recorded in the Western-style-diet fed animals compared to animals fed the nonpurified NTP-2000 diet ($P < 0.05$) with a much lower fat content suggests a tumor-promoting effect of dietary fat in Tg.NK mice. This is in accordance with epidemiological and animal studies demonstrating a positive correlation between dietary fat intake and cancer (61–63). However, a promoting effect of Prevastein in form of increased tumor number per tumor-bearing mouse was also observed in Tg.NK mice on the nonpurified NTP-2000 diet with considerably lower fat content (Phyto-prevent meeting, Jena, June 2002, data not published; see Ref. 34), suggesting that dietary fat is not a prerequisite for Prevastein to promote mammary tumorigenesis, although its effect in this study may be mediated in synergy with alterations induced by dietary fat.

The determination of the activity of cytochrome (P450) enzymes GST and QR in the liver was included in this study because one of the proposed health beneficial effects of flavones is their potential anticarcinogenic properties connected to the ability to modulate phase I and phase II metabolism enzymes. Genistein has been shown to inhibit CYP450 enzymes in vitro and induce GST activity in vivo, suggesting a role in inhibiting procarcinogen activation and enhancing detoxification of carcinogens (45,64). As no alteration of activity of the selected hepatic metabolism enzymes was recorded under the conditions of this bioassay, we suggest that the soy isoflavones are not directly involved in regulation of the activity of these enzymes in Tg.NK mice. This finding is in agreement with other studies (65,66).

It has been suggested that dietary genistein enhances the activities of antioxidant enzymes in vivo and that this effect contributes to the chemopreventive mechanism of genistein (67). The absence of difference of GPx activity as a marker for redox status in RBCs of Prevastein-exposed versus control animals does not support an in vivo antioxidant potential of soy isoflavones in this model.

In conclusion, these findings in Tg.NK mice indicate that addition of Prevastein as a source of soy isoflavones to the Western-style diet has no effect on the activity of selected hepatic phase I and phase II enzymes or on the activity of antioxidant enzymes in erythrocytes. Analysis of the mammary gland indicates that soy isoflavones can induce epithelial proliferation at an early age, manifested by increased branching frequency and apoptotic activity, which may result in a more quiescent epithelium in adults evident by fewer TEBs and lower apoptotic activity. Exposure to dietary soy isoflavones added to a Western-style diet does not alter latency or growth rate of tumors in this bioassay; however, the

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### Table 4. Characterization of Tg.NK Mouse Liver Microsomes by Use of Alkylresorufin

<table>
<thead>
<tr>
<th>Activity (CYP450)</th>
<th>Postnatal Week 6 Isoflavones (mg/kg Western-Style Diet)</th>
<th>Postnatal Week 10 Isoflavones (mg/kg Western-Style Diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>EROD (1A)</td>
<td>364 ± 67</td>
<td>368 ± 85</td>
</tr>
<tr>
<td>MROD (1A)</td>
<td>348 ± 43</td>
<td>407 ± 65</td>
</tr>
<tr>
<td>PROD (2B)</td>
<td>72 ± 15</td>
<td>80 ± 11</td>
</tr>
<tr>
<td>BROD (3A)</td>
<td>167 ± 24</td>
<td>175 ± 29</td>
</tr>
</tbody>
</table>

* $a$: Abbreviations are as follows: CYP450, cytochrome P450; EROD, ethoxyresorufin-O-deethylase; MROD, methoxy-O-deethylase; PROD, pentoxyresorufin-O-deethylase; BROD, benzoyloxy-O-deethylase. Data is given as the mean of triplicate experiments ± SD of samples from five to six animals per group at postnatal Week 6 and 10. The predominant CYP subgroups involved in the dealkylation reactions are listed in parentheses (45).
high Prevastein dose is associated with increased number and size of mammary tumors. This adverse effect, although moderate, should be considered, keeping in mind the wide use of soy isoflavones in commercially available health products especially when consumed by individuals with cancer.

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