In utero exposure to maternal diets containing soy protein isolate, but not genistein alone, protects young adult rat offspring from NMU-induced mammary tumorigenesis

Ying Su1,2, Renea R. Eason1, Yan Geng1,2, S. R. Tilt1, Thomas M. Badger1,2, and Rosalía C. M. Simmen1,2,*

1Arkansas Children’s Nutrition Center and 2Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, 1120 Marshall Street, Little Rock, AR 72202, USA

*To whom correspondence should be addressed. Tel.: +1 501 364 2849; Fax.: +1 501 364 3161; Email: simmenrosalia@uams.edu

The linkage of nutrition and cancer prevention is an intriguing concept that is gaining widespread support. Here, we investigated the influence of developmental context on dietary protection against tumorigenesis initiated by the direct-acting carcinogen 1-N-methyl-N-nitrosourea (NMU), and examined potential mechanisms underlying these effects. Rats were exposed only in utero or for lifetime to American Institute of Nutrition-93G diets made with casein (CAS), soy protein isolate (SPI) or CAS supplemented with genistein (GEN). Mammary glands of post-natal day (PND) 50 rats prior to NMU administration were examined for apoptotic status, pro-apoptotic gene expression and immunoreactive phosphatase and tensin homolog deleted on chromosome ten (PTEN) and epithelial cadherin (E-cadherin) levels, whereas mammary tumor parameters were evaluated 99 days post-NMU. Animals exposed only in utero to SPI had increased tumor latency, decreased tumor multiplicity and lower higher grade tumors, than those fed CAS. In utero exposure to GEN resulted in similar tumor parameters as the CAS group, whereas lifetime SPI exposure decreased tumor incidence that was not mimicked by in utero exposure alone. Mammary glands of PND50 rats fed lifetime SPI had increased terminal end bud apoptotic status and PTEN expression, than the other diet groups. Rats exposed only in utero to SPI or GEN had higher membrane E-cadherin in mammary structures than those lifetime-fed CAS or SPI. Thus, limited exposure during gestation to SPI can positively influence resistance to chemically induced mammary tumorigenesis later in life. Preventative strategies against mammary and other types of cancer might be uncovered by refinement of the developmental window for dietary factor exposure.

Introduction

Cancer is a genetic disease elicited by mutations in tumor suppressors, oncogens and DNA instability genes, allowing for the clonal expansion of damaged cells leading to neoplasia (1). Breast cancer is the second leading cause of death and the most common malignancy among women, with >200 000 new cases predicted in 2006 in the USA alone. The overall survival rate for breast cancer has increased for lifetime to American Institute of Nutrition-93G diets made with casein (CAS), soy protein isolate (SPI) or CAS supplemented with genistein (GEN). Mammary glands of post-natal day (PND) 50 rats prior to NMU administration were examined for apoptotic status, pro-apoptotic gene expression and immunoreactive phosphatase and tensin homolog deleted on chromosome ten (PTEN) and epithelial cadherin (E-cadherin) levels, whereas mammary tumor parameters were evaluated 99 days post-NMU. Animals exposed only in utero to SPI had increased tumor latency, decreased tumor multiplicity and lower higher grade tumors, than those fed CAS. In utero exposure to GEN resulted in similar tumor parameters as the CAS group, whereas lifetime SPI exposure decreased tumor incidence that was not mimicked by in utero exposure alone. Mammary glands of PND50 rats fed lifetime SPI had increased terminal end bud apoptotic status and PTEN expression, than the other diet groups. Rats exposed only in utero to SPI or GEN had higher membrane E-cadherin in mammary structures than those lifetime-fed CAS or SPI. Thus, limited exposure during gestation to SPI can positively influence resistance to chemically induced mammary tumorigenesis later in life. Preventative strategies against mammary and other types of cancer might be uncovered by refinement of the developmental window for dietary factor exposure.

Materials and methods

Animal studies

Animal protocols were approved by and in compliance with guidelines from the University of Arkansas for Medical Sciences Committee on the Use and Care of Animals. Time-mated Sprague–Dawley rats were purchased from Charles Rivers Laboratories (Wilmington, MA) and housed individually in polycarbonate cages under conditions of 24°C, 40% humidity and a 12 (light–dark) cycle. Rats at gestation day 4 were randomly assigned to semi-purified isocaloric diets made according to the American Institute of Nutrition-93G formula (20), with corn oil substituting for soybean oil and containing the following as sole protein source: (i) CAS (New Zealand Milk Products, Santa Rosa, CA); (ii) CAS to which GEN was added in the aglycone form (250 mg/kg feed) (Sigma Chemical Co., St Louis, MO) and (iii) SPI (Solae Company, St Louis, MO). The latter contained 394 ± 16 mg total isoflavones/kg diet, including 216 ± 2 mg/kg GEN and 160 ± 6 mg/kg daidzein, both expressed as aglycone equivalents (18,19). Animals were provided food and water ad libitum. Figure 1

Abbreviations: CAS, casein; DCIS, ductal carcinoma in situ; DE, ductal epithelium; DMBA, 7,12-dimethylbenz(a)anthracene; E-cadherin, epithelial cadherin; GEN, genistein; IDC, invasive ductal carcinoma; NMU, N-methyl-N-nitrosourea; PND, post-natal day; PTEN, phosphatase and tensin homolog deleted on chromosome ten; SPI, soy protein isolate; TEB, terminal end bud.

© The Author 2006. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org
summarizes the four dietary regimens evaluated. At delivery [post-natal day (PND) 0], pups from dams fed SPI throughout pregnancy were pooled and randomly assigned to these dams for suckling. The dams were divided into two diet groups as follows: one group was continued on SPI (SPI group), whereas the other group was switched to the CAS diet (SPI-CAS group). Pups assigned to dams fed CAS + GEN throughout pregnancy and switched to CAS during lactation represented a third diet group (GEN–CAS group). A fourth diet group consisted of pups from dams fed CAS during pregnancy and through lactation. Each dam was assigned 10 pups (five per sex) for suckling. Female pups were weaned at PND21 to the same diets as their assigned dams for lactation and were continued on these diets throughout the study. At PND50, female pups (n = 10 each for CAS, SPI and SPI-CAS; n = 5 for GEN–CAS) were killed and the inguinal mammary gland (gland number 4) pair was removed. Portions of the left gland were fixed for paraffin embedding, whereas the right gland was immediately homogenized in TriZol (Invitrogen, Carlsbad, CA) for RNA extraction. Male pups were used in unrelated studies.

Caricinogen administration and tumor analysis
At PND51, the remaining female pups were administered the alkylating agent N-methyl-N-nitrosourea (NMU, Lot #ASL-701, Ash Stevens, Detroit, MI) freshly dissolved in 0.9% saline solution (pH 4.6) at a dose of 50 mg/kg body wt, via jugular vein injection. Rats were weighed weekly and beginning at 2 weeks after NMU treatment were palpated on a weekly basis for mammary tumors. For each rat, the initial detection date of palpable tumors, the subsequent detection dates of new tumors and their locations and the total tumor numbers were recorded. Rats from all diet groups were killed when 80% of CAS-fed rats showed palpable tumors (99 days post-NMU). Tumors from each rat were dissected, weighed and preserved in 10% neutral buffered formalin. Tissue sections from the largest tumor from each tumor-bearing rat were stained with hematoxylin–eosin and histologically classified by a board-certified pathologist, as described in previous studies from this group (19).

Immunohistochemistry
Mammary glands were fixed overnight in 10% neutral buffered formalin, dehydrated with gradient alcohol and embedded in paraffin. Tissue sections were stained using the following: (i) anti-phospho-p53Ser46 (P-p53Ser46, Cell Signaling Technology, Danvers, MA); (ii) anti-PTEN (A2B1, Santa Cruz Biotechnology, Santa Cruz, CA) and (iii) anti-epithelial cadherin (E-cadherin) (BD Transduction Laboratories, San Jose, CA), following previously described protocols from this group (11, 21) or the manufacturer’s instructions. Antigen retrieval in Citra Plus (Biogenex, San Ramon, CA) and incubation with blocking solution (Casblock, Zymed, San Francisco, CA) to minimize non-specific binding were also described previously (21). Immunoperoxidase staining was developed with 3,3’-diaminobenzidine chromagen (Dako Corp, Carpinteria, CA), and slides were counterstained with hematoxylin or methyl green. For PTEN and P-p53Ser46, four randomly chosen fields (×200) per slide per rat (n = 3–4 individual rats per diet) were evaluated for numbers of dark brown color-staining cells, indicating positive expression. For E-cadherin, 10 randomly chosen fields per slide per rat (n = 4–5 individual rats per diet) were independently scored for immunostaining intensities by three laboratory members who were presented with the slides in a blinded fashion. A scoring range of 1 (moderate staining) to 3 (highest staining) was used.

RNA extraction and quantitative real-time reverse transcription—polymerase chain reaction
Total RNA was isolated from mammary glands using TriZol reagent according to the manufacturer’s instructions. Integrity of isolated RNAs was confirmed using the RNA 6000 Nano LabChip kit with the Agilent Bioanalyzer System (Agilent Biotechnologies, Palo Alto, CA). The cDNA was generated using 1 μg total RNA with random hexamers and MultiScribe Reverse Transcriptase kit (Bio-Rad Laboratories, Hercules, CA). The primer sequences for rat PTEN, Bax, p21 and 18S have been described in previous studies (11, 22). Polymerase chain reaction mix (25 μl) contained 10 nM of primers, 10 ng of cDNA and 2× SYBR Green PCR Master Mix (Bio-Rad Laboratories). Polymerase chain reaction conditions were as follows: 50°C, 2 min and 95°C, 1 min and 40 cycles of 95°C, 15 s and 60°C, 1 min. Relative levels of messenger RNA were normalized to 18S ribosomal RNA and expressed as arbitrary units.

Results
Diet effects on mammary tumor parameters
Female offspring exposed to lifetime CAS or SPI served as negative and positive controls, respectively, for the mammary tumor protective effects of lifetime-fed diets (19). The appearance of palpable tumors occurred earliest with the CAS group, with 50% of rats showing at least one tumor by 63 days post-NMU (Figure 2A). In contrast, for the other groups fed SPI or GEN, the appearance of at least one tumor in 50% of the rats was not observed until 71 (GEN–CAS), 77 (SPI–CAS) and 78 (SPI) days post-NMU, respectively (Figure 2A). Thereafter, whereas rats in the SPI–CAS and GEN–CAS groups rapidly accumulated tumors, reaching tumor incidence comparable with those of the CAS group by 99 days post-NMU, the number of tumor-bearing rats in the SPI group remained relatively constant (Table I and Figure 2A). At the conclusion of the study (PND149), the SPI group had ~20% lower tumor incidence (P = 0.062) compared with the other diet groups.

Analysis of tumor multiplicity among the diet groups indicated lower tumor numbers per tumor-bearing rat in the SPI–CAS group, relative to the other diet groups (Table I). Indeed, whereas only 2% of the tumor-bearing rats in the SPI–CAS group showed more than seven tumors on average, 20% of those in the SPI and GEN–CAS groups and 45% in the CAS group belonged in this category (Figure 2B).

The largest mammary tumor from each rat of all diet groups was histologically analyzed and scored as benign (normal mammary tissues with fibrosis), precancerous (intra-ductal proliferation) or cancerous [ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC)] lesions. The incidence of benign and precancerous lesions did not differ among the diet groups (Table I). Further, the diet groups showed comparable incidence (percentage) of tumors designated as cancerous lesions (DCIS + IDC) (Table I). However, the SPI group showed numerically higher percentage of the more invasive tumor (IDC), whereas the SPI–CAS group had the lowest percentage of this tumor grade (Table I) among the other three groups. In particular, SPI–CAS–fed rats exhibited a greater percentage of DCIS (60%) than IDC (30%) when compared with those fed lifetime SPI (P < 0.004).

Diet effects on mammary apoptosis
Expression of Tp53 phosphorylated at Serine 46 (immunoreactive P-Tp53Ser46) was used as a readout for apoptosis since phosphorylation of Tp53 at this serine residue is a hallmark of apoptotic pathway activation (23). Mammary terminal end buds (TEBs) of PND50 rats fed lifetime SPI had higher numbers of cells showing Tp53Ser46 immunoreactivity relative to those of the other diet groups (P < 0.001),
which exhibited comparable levels (Figure 3). Ductal epithelium (DE) structures showed minimal immunostaining for Tp53<sub>Scr</sub>, albeit the trend (higher for SPI than for all the other diet groups; \( P = 0.067 \)) was similar to that observed for TEBs.

**Diet effects on mammary gene expression**

The transcript levels of the pro-apoptotic genes Bax, p21 and PTEN did not differ in mammary tissues of rats from the different diet groups (data not shown). Consistent with our previously published studies (11), lifetime dietary exposure to SPI (SPI group) had no effect on mammary expression of p21 and Bax, relative to those of rats fed the control diet CAS. Dietary exposure to SPI only in utero (SPI–CAS group) mimicked the patterns of p21 and Bax expression of the SPI group. In contrast, whereas lifetime exposure to GEN increased the mammary transcript levels of p21 and Bax, relative to those of CAS (11), in utero exposure only to GEN (GEN–CAS) did not affect the expression of these genes when compared with the CAS group.

**Diet effects on mammary PTEN protein levels**

The number of PTEN immunopositive cells was evaluated in mammary TEB and DE of PND50 rats exposed to lifetime SPI or only in utero (SPI–CAS and GEN–CAS). The number of cells with nuclear and cytoplasmic PTEN immunostaining was recorded separately for each mammary structure, since cellular localization of PTEN has been linked to distinct effects on cell cycle arrest and apoptosis (24). Expression of PTEN in TEB (quantified as numbers of PTEN immunopositive cells per structure) was significantly higher (\( P < 0.05 \)) for the SPI group relative to the two other diet groups, irrespective of whether nuclear or cytoplasmic PTEN immunoreactivity was evaluated (Figure 4A). A similar trend for DE was observed between SPI and the other two diet groups (Figure 4B), although the differences were not significant.

**Diet effects on E-cadherin expression**

PTEN is reported to form a complex with membrane-bound E-cadherin, a tumor suppressor, and β-catenin, leading to lower β-catenin activation (25) and decreased tumorigenesis (26). Because increased PTEN immunoreactivity in mammary structures is associated with lifetime dietary exposure to SPI, but not with in utero-only exposure to SPI or GEN (Figure 4), we evaluated E-cadherin distribution in mammary tissues of rats of the different diet groups by immunohistochemistry. E-cadherin localized at sites of cell–cell contacts in mammary TEB and DE and was not detected in the cytoplasmic compartment (Figure 5). Mammary TEB and DE of rats of the four diet groups showed greater membrane E-cadherin immunoreactivity in SPI–CAS and GEN–CAS groups relative to SPI and CAS groups, respectively. (Figure 5A–C).

**Discussion**

The present study evaluated the consequence of limited early developmental exposure (in utero) to diets containing the soy isoflavone GEN on mammary tumor development in a rat model of mammary carcinogenesis. We report here that for young adult female rats exposed to the carcinogen NMU, in utero-only exposure to SPI (SPI–CAS group) was associated with increased tumor latency, decreased tumor multiplicity and lower incidence of more invasive tumors (e.g. IDC), relative to the control diet CAS. Moreover, in utero exposure to supplemental GEN alone (GEN–CAS group) largely mimicked the tumor parameters observed for the CAS group, with the exception of higher tumor latency. Further, lifetime dietary SPI exposure (SPI group) decreased tumor incidence that was not mimicked by in utero exposure only to SPI (SPI–CAS) and GEN–CAS (GEN–CAS group).

**Table 1. Mammary tumor parameters and developmental stage of dietary exposure**

<table>
<thead>
<tr>
<th>Rats/diet</th>
<th>Tumor incidence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tumor multiplicity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Benign</th>
<th>I/DP&lt;sup&gt;c&lt;/sup&gt;</th>
<th>DCIS&lt;sup&gt;d&lt;/sup&gt;</th>
<th>IDC&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAS</td>
<td>47</td>
<td>80.85%</td>
<td>4.4 ± 0.533</td>
<td>1 (2.6%)</td>
<td>2 (5.3%)</td>
<td>15 (39.5%)</td>
</tr>
<tr>
<td>SPI</td>
<td>44</td>
<td>61.36%</td>
<td>3.3 ± 0.492</td>
<td>2 (7.4%)</td>
<td>0 (0%)</td>
<td>7 (25.9%)</td>
</tr>
<tr>
<td>SPI–CAS</td>
<td>47</td>
<td>85.11%</td>
<td>2.2 ± 0.231</td>
<td>2 (5%)</td>
<td>2 (5%)</td>
<td>24 (60.0%)</td>
</tr>
<tr>
<td>GEN–CAS</td>
<td>39</td>
<td>82.05%</td>
<td>3.6 ± 0.502</td>
<td>4 (12.5%)</td>
<td>0 (0%)</td>
<td>12 (37.5%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Tumor incidence = percentage of tumor-positive rats at 99 days post-NMU.
<sup>b</sup>Tumor multiplicity = average number of tumors/tumor-bearing rat.
<sup>c</sup>I.DP, intra-ductal proliferation.
<sup>d</sup>Number of rats with designated tumor grade (percentage of the total tumor-bearing rats).
<sup>e</sup>\( P < 0.05 \), relative to CAS (by one-way analysis of variance); and **\( P = 0.004 \), relative to SPI (by Fisher’s exact test).
exposure alone, and which was distinctly associated with increased mammary TEB apoptotic status and PTEN expression prior to NMU administration. Finally, increased membrane E-cadherin localization in mammary structures prior to NMU insult could partly underlie the tumor protective effects of in utero exposure to soy diets. Taken together, these results suggest that restricted dietary exposure to SPI at a very early developmental window can attenuate mammary tumor progression later in life, and which may involve the induction of mechanisms for the maintenance of the E-cadherin adhesion complex (27). Moreover, these findings provide evidence for the distinct effects of diet on signaling pathways associated with the carcinogenic process, namely PTEN and E-cadherin, and set the groundwork for further identification of bioactive compounds in soy foods other than GEN, with mammary tumor protective effects (28). As regards the latter, the soy isoflavone daidzein is a likely candidate, given its ability to delay 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumor development in the mouse mammary tumor virus-neu mouse model (29) and to inhibit DNA adduct formation in mammary glands of mice administered DMBA (30).

Our studies were conducted to address the developmental context of the cancer preventative effects of dietary SPI, as gleaned from epidemiological studies of Asian women with relatively low incidence of breast cancer (4,5,28). Since soy food consumption in this population occurs mostly for lifetime, the consequence of limited exposure is restricted to pregnancy. An important practical implication of these studies is that the period of dietary intake can be manipulated for maximal health benefits.

The lower tumor multiplicity and tumor grade with in utero-only exposure to dietary SPI, relative to that of lifetime SPI exposure, suggest the ability of SPI to influence fetal mammary gland development with positive outcome against adult risk of mammary cancer. In rodents, late gestation (6 to 7 days before birth), when primary ducts begin to form at the epithelial bud site in response to signals from the surrounding fat pad, is considered a critical period of mammary gland development (33,34). The comparable developmental period for human fetal breast occurs earlier at around 12–14 weeks of embryonic life (34,35). Our data indicate that in utero events leading to the induction of E-cadherin-mediated cell–cell adhesion by SPI in young adult (PND50) mammary gland may contribute to tumor protection (27,36), and raise the possibility of epigenetic changes involving a subset of mammary epithelial cells that are preserved (imprinted) during development (37). Although in utero-only GEN exposure similarly elicited induction of membrane E-cadherin expression at a level comparable with that of SPI–CAS in PND50 mammary glands, the lack of a significant mammary tumor protection with this dietary
regimen indicates the contribution of additional ‘safeguard’ mechanisms induced by SPI, independent of GEN or related metabolites. These may involve perturbations of the developmental programming with positive effects on immune and DNA repair system maturation, ductal differentiation, glandular structure formation and tumor suppressor methylation. Finally, the inability of lifetime SPI exposure to mimic the up-regulation of E-cadherin expression by in utero exposure implies that events occurring post-natally may alter these early effects. In this regard, activation of other signaling pathways during the peripubertal growth of the mammary tissue, another critical developmental window (34), may confer the increased resistance noted for lifetime SPI diet on tumor development. Based on our previous report (11), and which we confirmed here, we posit that one such pathway involves the activation of PTEN-mediated apoptosis, leading to the early elimination of damaged (mutated) cells that can give rise to tumorigenic cells. Consistent with this, we demonstrated higher levels of cytoplasmic PTEN immunoreactivity, which has been linked to regulation of apoptosis (24), in TEB and to a lesser extent in DE, for the SPI group relative to the SPI–CAS and GEN–CAS groups. Although we are unable to provide a molecular mechanism describing post-natal activation of PTEN signaling by SPI at the present time, we do not know the distinct chronology of events leading to the up-regulated expression of PTEN and E-cadherin at PND50. Similarly, the significance of the reported association of PTEN with the E-cadherin–catenin complex reported previously for pancreatic carcinoma cells (25) is unclear in the context of the mammary epithelium. Clearly, the functional effects of soy proteins and individual biologically active components in mediating PTEN and E-cadherin expression to endow mammary epithelial cells with resistance to carcinogenic agents must be further evaluated.

Fig. 5. E-cadherin expression in mammary glands of PND50 rats of the different diet groups. Mammary sections representing individual animals (n = 4–5) of each diet group were analyzed. Representative micrographs of TEB (A) and DE (B) of PND50 rat mammary glands immunostained with anti-E-cadherin antibody. Positive staining (dark brown color; indicated by arrowheads) was localized to membranes of mammary epithelial cells. (C) Graphical representation of immunostaining intensities (means ± SEMs), with representative score of 1 for CAS–TEB and of 3 for GEN/CAS-TEB. Means with different superscripts differed at P < 0.05.
In summary, our results show that limited exposure to SPI in utero can elicit significant mammary tumor protective effects that may have increased advantage over that of lifetime exposure. We further show that exposure to supplemental GEN at a similar developmental window does not result in mammary tumor protection comparable with that of SPI. Additionally, we identified signaling pathways involving the tumor suppressors PTEN and E-cadherin as potential mechanisms underlying the tumor protective effects of dietary soy proteins at two developmental exposure contexts. We suggest that strategic approaches for the prevention of mammary and other types of cancer might be uncovered by careful refinement of the developmental window for exposure to specific dietary factors.

Acknowledgements
The authors thank the following for their contributions to this work: Dr Frank A. Simmen for helpful discussions during the course of the study and critique of the manuscript; Matt Ferguson, Trae Pittman and Tammy Dallari for assistance with the animal work; Dr Sohelia Korourian (University of Arkansas for Medical Sciences) for histopathological analyses of mammary tumors; Pamela Treadaway and Mark Robinette for assistance with data collection and members of this laboratory for help in scoring immunopositive slides. Funds for this research were provided by the United States Department of Agriculture-Current Research Information System-6251-5100002-06S to the Arkansas Children’s Nutrition Center.

Conflict of Interest Statement: None declared.

References

Received June 17, 2006; revised November 22, 2006; accepted November 27, 2006

In utero SPI exposure and mammary tumor protection