The soy isoflavone genistein promotes apoptosis in mammary epithelial cells by inducing the tumor suppressor PTEN

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The isoflavone genistein (GEN), a biologically active component of soy foods, is associated with reduced breast cancer risk in women who consume soy-rich diets. GEN has been reported to influence many biological processes, of which suppression of cell proliferation and stimulation of apoptosis are considered to be the major pathways underlying its inhibition of tumorigenesis. This study evaluated the mechanism by which diets containing GEN promote mammary epithelial cell death. We report that mammary glands of young adult female rats exposed from gestation day 4 to postnatal day 50, to AIN-93G diets containing as sole protein source, casein (CAS) supplemented with GEN, or soy protein isolate (SPI⁺) had increased apoptosis, relative to rats fed CAS diet devoid of GEN. Mammary gland proliferation was unaffected by diet. The increased apoptotic index in mammary glands of GEN and SPI⁺ rats was accompanied by increased levels of the tumor suppressor protein PTEN (phosphatase and tensin homolog deleted in chromosome ten), albeit enhanced mammary expression of the pro-apoptotic p21, Bax and Bok genes was observed only in GEN-fed rats. GEN-induced apoptosis in MCF-7 cells was concomitant with increased PTEN expression, and this was abrogated by PTEN siRNA. MCF-7 cells treated with serum from GEN- or SPI⁺-fed rats had increased apoptosis as well as increased levels of the PTEN transcript. PTEN siRNA attenuated the increased apoptotic response of MCF-7 cells to serum from rats fed SPI⁺ or GEN, although the inhibition to basal (CAS serum) apoptotic levels was achieved only for cells treated with GEN serum. Decreased p21 and Bok gene expression accompanied the inhibition of apoptosis by PTEN siRNA. Data implicate PTEN in the induction of apoptosis by GEN and suggest that the promotion of apoptosis leading to inhibition of tumorigenesis in vivo by diets containing GEN may also involve the distinct activities of yet unknown GEN metabolite(s) and/or other systemic factors induced by GEN.

Introduction

Breast cancer affects one of nine women in their lifetime and is the second leading cause of cancer-related deaths, next to lung cancer, in the US female population (1). Since this is a heterogenous disease that has been suggested to arise as a result of both genetic and epigenetic modifications (2–4), there is no known single effective strategy for its prevention or treatment. However, diet has been proposed to constitute an important determinant for decreasing risk of breast cancer (5–7). Evidence to support this include recent epidemiological studies indicating that the incidence of the disease is significantly lower in Asian women whose intake of soy products, especially during adolescence, is 20–50 times more than by American females (8,9). Soy products are a rich source of phytoestrogens of which the primary soy isoflavone, genistein (GEN), is considered to play a key role in breast cancer protection (10–12). GEN exhibits estrogen agonist and antagonist activity through estrogen receptor (ER)-α and ER-β mediated pathways (13,14), that is dose- and cell context-dependent (15). It is also an inhibitor of protein tyrosine kinases, whose actions are crucial to the control of cellular growth and apoptosis (16). More recent studies using animals and human cells suggest that multiple regulatory pathways could be influenced by GEN, including through mediation of stress responses (17); apoptosis (18,19); HER-2/neu (20) and epidermal growth factor receptor (21) signaling; and proteosomal activity (22). Normal cells differ from cancer cells in their ability to undergo apoptosis when cellular DNA damage is initiated (23). Consequently, the genetic and biochemical mechanisms that confer activation of the ‘death’ machinery and associated ‘death’ genes have been the subject of intense investigations, in an effort to develop effective strategies for human cancer prevention and therapy (24,25). Studies from the last few years have established a critical role for the dual lipid/protein phosphatase PTEN (phosphatase and tensin homolog deleted in chromosome ten) in tumor suppression (26–29). Loss of or reduction in PTEN expression has been reported in a wide array of human cancers, including those of the uterus, prostate, lung, mammary, and ovary (30–32), and is associated with poor clinical outcome (33). Accordingly, in mouse models of PTEN deficiency, increased susceptibility to mammary neoplasia (34) and precocious mammary development (35) were observed. The best elucidated function of PTEN as a tumor suppressor relates to its negative regulation of the PI3-kinase/Akt pathway by virtue of its ability to dephosphorylate phosphatidylinositol 3,4,5-triphosphate (36). Constitutive Akt activity as a result of loss of PTEN function, leads to increased proliferation and reduced apoptosis, both of which are hallmarks of tumorigenic potential. Other recently defined

Abbreviations: AIN, American Institute of Nutrition; CAS, casein; DMBA, 7,12 dimethylbenz[a]anthracene; GD, gestation day; GEN, genistein; PCNA, proliferating cell nuclear antigen; PI3-K, phosphatidylinositol-3-kinase; PND, postnatal day; PTEN, phosphatase and tensin homolog deleted in chromosome ten; qPCR, quantitative real-time polymerase chain reaction; SEM, standard error of the mean; siRNA, small interfering RNA; SPI⁺, soy protein isolate; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick-end labeling.
tumor-suppressive mechanisms of PTEN include inhibition of the growth factor-activated ras/mitogen activated protein kinase pathways (37), dephosphorylation of focal adhesion kinase leading to inhibition of cell migration (38), and recruitment of p53 in response to DNA damage (39).

Our group and others have used rat models of chemically-induced mammary tumorigenesis to evaluate the effects of diet during early development on adult risks of mammary cancer (40–42). These studies demonstrated inhibition of DMB-42 and NMU-induced (43) mammary tumor development in rats lifetime-fed soy protein isolate (SPI1), a mimic of the ‘Asian diet’ rich in isoflavones, relative to those fed the control casein (CAS) diet. The underlying mechanism(s) for these effects of SPI1 remains poorly defined, despite observations that alterations in differentiation, proliferation, apoptosis, and carcinogenic metabolic pathways may be involved (15,44–47). Moreover, soy products contain a host of yet unidentified peptides as well as non-protein components, including saponins, phytic acid, lignans and other phytochemicals, whose individual and cumulative biological effects are unknown.

In the present study, we investigated the mechanism whereby the soy isoflavone GEN exerts its protective effects against mammary tumorigenesis in vivo (10–12). We demonstrate that GEN enhances mammary cell apoptosis by increasing the expression of the tumor suppressor/pro-apoptotic protein PTEN as well as the pro-apoptotic genes Bok, Bak, and p21. We also show that, the natural dietary source of GEN, mimics GEN-induced apoptosis, albeit other signaling pathways in addition to that of PTEN, may be involved in this response. Further, we provide evidence for the regulation of systemic pro-apoptotic factors whose actions involve PTEN signaling, by diets containing GEN. Our observations provide mechanistic insights into the role of dietary GEN in the inhibition of mammmary tumorigenesis.

Materials and methods

Animals
Procedures for animal care and treatments followed the guidelines approved by the University of Arkansas for Medical Sciences Animal Care and Use Committee. Time-mated Sprague–Dawley rats, purchased from Charles Rivers Laboratories, Inc. (Wilmington, MA) were housed individually in polycarbonate cages under conditions of 24°C, 40% humidity, and a 12-h light–dark cycle. Rats at gestation day (G–D) 4 were randomly assigned to one of three semi-purified isolocaloric diets made according to the AIN-93G formulation (48), with corn oil substituting for soybean oil and containing as sole protein source: (i) casein (CAS; New Zealand Milk Products, Santa Rosa, CA); (ii) CAS to which was added genistein in the aglycone form (SPI1), a mimic of the ‘Asian diet’ rich in isoflavones, relative to those fed the control casein (CAS) diet. The underlying mechanism(s) for these effects of SPI1 remains poorly defined, despite observations that alterations in differentiation, proliferation, apoptosis, and carcinogenic metabolic pathways may be involved (15,44–47). Moreover, soy products contain a host of yet unidentified peptides as well as non-protein components, including saponins, phytic acid, lignans and other phytochemicals, whose individual and cumulative biological effects are unknown.

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Immunohistochemistry
Mammary glands were fixed overnight in 10% buffered-formalin, embedded in paraffin, and sectioned. The procedures for antigen retrieval in Citra Plus (Biogenex, San Ramon, CA), and incubation with blocking solution (Cas block; Zymed, San Francisco, CA) to minimize non-specific binding were previously described (49). Primary antibodies used were anti-proliferating cell nuclear antigen (PCNA, clone PC10; Dako Corp., Carpinteria, CA), and anti-PTEN (A2B1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoperoxidase staining was developed with 3,3′-diaminobenzidine chromagen (Dako Corp.), and slides were counterstained with hematoxylin. Four randomly chosen fields (×200) per slide per rat, with two slides for each tissue block from 4–5 individual rats per diet, were analyzed for presence of dark brown color-staining cells, indicating positive expression. The proliferation index was calculated as a percent of PCNA-positive cells relative to the total number of cells counted for each mammary structure (terminal end bud, TEB; lobules, LOB; ductal epithelium, DE). PTEN (cytoplasmic and nuclear) expression was determined by counting the number of positive cells per structure.

Terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick-end labeling (TUNEL) assay to detect apoptotic cells was performed following the manufacturer’s instructions (Oncogene, La Jolla, CA), except that incubation with TdT was carried out for 1 h at 37°C. TUNEL-positive cells were counted from three randomly selected fields at ×200 per slide, and two slides were evaluated for each tissue block from four individual rats per diet.

RNA extraction and quantitative real-time RT–PCR (qPCR)
Total RNA was isolated from mammary glands using Trizol reagent (Invitrogen, Carlsbad, CA). Integrity of isolated RNAs was confirmed using the RNA 6000 Nano LabChip kit with the Agilent Bioanalyzer System (Agilent Biotechnologies, Palo Alto, CA). Total RNA (1 μg) was reverse-transcribed using random hexamers and multi-scribe reverse transcriptase in a two-step RT–PCR reaction, following the manufacturer’s instructions (Bio-Rad Laboratories, Hercules, CA). Primers for PCR were designed to span intron/exon junctions ( PrimerExpress; Applied Biosystems, Foster City, CA) to minimize amplification of residual genomic DNA. The primer sequences for rat Bax, Bcl2, and Bok are (sense and anti-sense, respectively): Bax (5′-AGGCCGAATTTGCGGATGAA3′; 5′-GCTGCCAACCAGGAAAGAC3′); Bcl2 (5′-GGAGGATTGTGGCTCTTCTGG3′; 5′-GCCGGTTACAGTACAGTCACT-3′), and Bok (5′-GTCCCGGCTATCCGGAAT3′; 5′-CCCATGTGATACCTGCTCAGA3′); the primer pair for p21 was previously described (49). PCR mix (25 μl) contained optimal concentrations (10 nM) of primers, 10 ng of cDNA, and 2× SYBR Green PCR Master Mix (Bio-Rad Laboratories). The conditions for qPCR and determination of relative transcript levels were described previously (49). Relative gene expression was calculated with 18S rRNA as the internal control and was expressed as arbitrary units.

Cell culture, treatments and assays
Human breast cancer cells (MCF-7) were obtained from American Type Culture Collection (ATCC; Manassas, VA) and cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (GIBCO, Carlsbad, CA) in a 5% CO2:95% air at 37°C. For TUNEL, cells were seeded at a density of 1.2 × 10^5 cells per well on Immunofluorescence chamber slides (Fisher Scientific, Inc.). After 1–2 h incubation, cells were washed with PBS and incubated in fresh DMEM containing 0.5% FBS. Treatments were sera pooled from PND50 rats lifetime-fed CAS (n = 6), SPI (n = 6) or GEN (n = 7) added at 1% final concentration. Twenty-four hours later, cells were rinsed with PBS, fixed in 4% paraformaldehyde for 30 min, washed with PBS, and successively incubated with 0.1% Triton X-100/PBS for 15 min and Proteinase K for 5 min. Cells were incubated with fluorescein-labeled TdT reagent (Oncogene) in a humidified chamber at 37°C for 1 h. Labeled nuclei were counted in three separate fields (×100) containing 200–300 cells each, using an Olympus IX–71 microscope with a standard fluorescence filter. Data are presented as the percent of labeled nuclei from the total number of cells counted.

The isolation and culture of mammary gland epithelial cells followed protocols described by Jeffrey Rosen’s laboratory (http://www.bcm.edu/rosenlab/ protocols) ( Baylor College of Medicine, Houston, TX), as adapted from an initial report by Pullan and Streuli (50). The abdominal mammary gland pair (number 4) from PND80 rats exposed to CAS diet beginning at gestation day 4 was used as source of epithelial cells. Mammary tissues from two rats were pooled, and equal aliquots of isolated cells were plated in six-well culture dishes in F12 medium containing gentamicin (10 μg/ml), insulin (10 μg/ml), hydrocortisone (2 μg/ml), epidermal growth factor (10 ng/ml) and 200 units of penicillin/streptomycin. After 48 h, the medium was changed to F12 containing 5% FBS and the above factors at one-half of their original concentrations. Cells were allowed to grow for another 3 days before treatments with CAS, SPI or GEN sera (1% final concentration) as described for MCF-7 cells, and were analyzed for PTEN gene expression. Experiments were repeated three times.
Transfection of PTEN small interfering RNAs (siRNAs)
MCF-7 cells were seeded at a density of 1 × 10^5 cells per well in 24-well culture plates and allowed to grow to 50–70% confluence prior to transfection. Cells were transfected with duplexed siRNA (100 nM) against human PTEN (sense: 5'-GACUGAAGCCGUCAUCGAU-3'; anti-sense: 5'-UCGUUAACGCCCUUCAAGCUU-3'; Ambion, Inc., Austin, TX) using Lipofectamine (Invitrogen) in DMEM containing 1% FBS. To evaluate the specificity of the siRNA transfection procedure, MCF-7 cells were transfected in parallel with duplexed siRNA against GAPDH (Ambion) or an irrelevant siRNA (Ambion), as positive and negative controls, respectively. After incubation for 5 h at 37°C, cells were treated with pooled sera from PND50 rats fed CAS, SPI+ or GEN (see above), added to a final concentration of 1%. Control cells received 1% FBS in lieu of rat serum. Cells were incubated for an additional 24 h and then processed for TUNEL assay as described above. Another set of cells was similarly treated with pooled CAS, SPI+ or GEN sera (1% final concentration), after transfection with PTEN siRNA (100 nM) or after mock-transfection. Cells were collected in TriZol reagent 24 h later. RNA extraction and analysis of transcript levels by qPCR followed the protocols described above. Human primers for PCR are (sense and anti-sense, respectively): (i) Bax (5'-CATGAGGCTGCAGAGGATGAT-3'; 5'-GGCGGTGTCATAATGTCTTTCA-3'); (ii) p21 (5'-CAGCGACCTTCTTCTACAC-3'; 5'-GAGAACCGGAAACCAGGACA-3'); (iii) PTEN (5'-GCTATGGGATTTCCTCAGCA-3'; 5'-GGCGGTGTCATAATGTCTTTCA-3'); and (iv) Bok (5'-TCTTCTTGACCAATCATAGGCAG-3'; 5'-CCACCGCATAAGGGACAC-3'). 18S RNA was used as the normalization control, and levels of RNA transcript were expressed as arbitrary units.

Statistical analysis
Data from in vitro studies were derived from at least 3 independent experiments and are presented as least-squares means ± SEM. Differences between diet groups were statistically analyzed by one-way or two-way ANOVA, followed by inspection of all differences between pairs of means by Tukey’s test. P ≤ 0.05 was considered statistically significant.

Results
Diet effects on mammary proliferation and apoptosis
The proliferative index of mammary structures from PND50 rats lifetime exposed to CAS, SPI+ and GEN diets was determined by PCNA staining of histological sections. As shown in Figure 1A, the patterns of cell proliferation were similar for mammary glands of CAS and SPI+ rats, with TEB showing greater PCNA immunoreactivity than LOB (CAS, P = 0.004; SPI+, P = 0.012) and DE (CAS, P < 0.001; SPI+, P = 0.006). The proliferation pattern for GEN group differed slightly, with PCNA immunoreactivity being highest in TEB and LOB, and lowest in DE (P < 0.05). For each mammary structure type, proliferation index was not affected by diet. In contrast, there was a significant increase (P < 0.05) in the numbers of apoptotic (TUNEL-positive) cells in mammary structures of SPI+ and GEN, relative to those of CAS rats (Figure 1B). However, while the numbers of TUNEL-positive cells in TEB were comparable for rats fed SPI+ and GEN diets, LOB and DE structures exhibited higher numbers of apoptotic cells (P < 0.05) in GEN than in SPI+ rats.

Diet effects on mammary PTEN expression
Given the role of the dual protein/lipid phosphatase PTEN in apoptosis (26,36), that loss of PTEN expression is associated with increased tumorogenesis in tissues including the mammary gland (31,33), and that mutations in the PTEN gene are linked to 20–30% lifetime risk of breast cancer (51), we evaluated the levels of PTEN protein in mammary structures as a function of diet. Mammary tissue sections were incubated with anti-PTEN antibody to quantify the numbers of PTEN immunopositive cells. PTEN was immunolocalized to cytoplasmic and nuclear compartments in TEB, LOB and DE structures of mammary glands (Figure 2A). The number of PTEN immunopositive cells in TEB did not differ with diet (Figure 2B). LOB and DE of rats fed SPI+ and GEN had increased numbers of PTEN immunopositive cells than in those fed CAS (P < 0.05), although for both structure types, these numbers did not differ between SPI+- and GEN-fed rats.

Effect of diet on mammary gene expression
Since SPI+ and GEN similarly induced mammary cell apoptosis in vivo (Figure 1B) concomitant with increased PTEN expression in LOB and DE (Figure 2), we next evaluated if the underlying apoptotic signaling pathway(s) downstream of PTEN was similar for both diets. To address this, we determined the expression levels of the apoptotic-associated genes Bax, Bcl2, p21 and Bok in mammary tissues of PND50 rats lifetime exposed to CAS, SPI+ or GEN diets. The mammary expression of all four genes did not differ between the CAS and
SPI+ groups (Figure 3A). By contrast, mammary expression of p21 ($P = 0.001$), Bax ($P = 0.02$), and Bok ($P = 0.03$) genes was higher for GEN than for CAS rats (Figure 3B), while that for Bcl2 was comparable ($P = 0.61$) between the two diets.

**Induction of mammary apoptosis by genistein involves PTEN**

We next used the mammary epithelial carcinoma MCF-7 cells as an *in vitro* system to evaluate a direct role for PTEN in mediating the observed induction of mammary epithelial cell apoptosis by GEN *in vivo* (Figure 1B). In this study, RNA interference (52) was used to inhibit the expression of PTEN, and resultant effects on GEN-induced apoptosis were evaluated by TUNEL. Two experiments were performed to initially define the optimal conditions for the interference assay. First, GAPDH siRNA was used as a positive control for the transfection protocol. The robust ‘knock-down’ in GAPDH mRNA expression (~80%), as shown in Figure 4A, confirmed the working conditions for siRNA transfection (see Materials and methods). Under these conditions, PTEN siRNA, when added at 100 nM final concentration suppressed basal PTEN expression by ~70% (Figure 4B); hence, this siRNA concentration was used in subsequent experiments (below). Second, a control siRNA (designated negative control) was assessed for its ability to inhibit both GAPDH and PTEN expression; its lack of effect on the expression of both genes indicated its utility as a negative control for the apoptosis experiments (Figure 4A and B).

MCF-7 cells were treated with pure GEN in the presence and absence of transfected PTEN siRNA, and PTEN expression levels were determined in RNAs prepared from these cells. GEN (2 μM) increased PTEN transcript levels, and this effect was lost when cells were incubated with PTEN siRNA during treatment (Figure 5A). The levels of apoptosis in GEN-treated
separately for CAS versus SPI by qPCR and normalized to 18S RNA. The analysis was carried out pooled from PND50 rats (CAS, cultured in the presence of serum (1% final concentration). Its direct effect on mammary epithelial cells, MCF-7 cells were

To investigate whether induction by GEN of mammary apoptosis occurs through other mechanism(s) in addition to factors present in serum, MCF-7 cells were treated with serum from CAS, SPI+ or GEN-fed rats, each added to a final concentration of 1%, in the presence and absence of PTEN siRNA. Consistent with the earlier results (Figure 6, above), GEN (P < 0.001) and SPI+ (P < 0.001) serum increased the percent of apoptotic cells, relative to CAS serum (Figure 7A and B). CAS serum alone had higher pro-apoptotic activity than 1% FBS. Cells transfected with PTEN siRNA (100 nM) and incubated with the test serum (CAS, SPI+ and GEN) had significantly reduced numbers of apoptotic cells than serum-treated, mock-transfected (without PTEN siRNA) cells (P = 0.05 for CAS; P < 0.001 for both SPI+ and GEN) (Figure 7A and B). Interestingly, while PTEN siRNA completely abolished the pro-apoptotic effect of GEN serum to the level of CAS serum, this was not observed for SPI+ serum. In the latter, the percent of apoptotic cells in cultures with added PTEN siRNA was significantly higher (by 50%) than in those

cells were also compared in the presence and absence of transfected PTEN siRNA. There was a dose-dependent increase in the percent of apoptotic cells when GEN was added at increasing concentrations to cultures (10 μM > 2 μM > 0 GEN; P < 0.001) (Figure 5B). Addition of PTEN siRNA abrogated the apoptosis-inducing effect of GEN in these cells.

Effects of rat sera on mammary cell apoptosis and PTEN gene expression

To investigate whether induction by GEN of mammary apoptosis in vivo occurs through other mechanism(s) in addition to its direct effect on mammary epithelial cells, MCF-7 cells were cultured in the presence of serum (1% final concentration) pooled from PND50 rats (CAS, n = 6; SPI+, n = 6; GEN, n = 7) lifetime exposed to dietary CAS, SPI+ or GEN, and the numbers of apoptotic cells were measured by TUNEL. As shown in Figure 6A, the percent of apoptotic cells was significantly increased by 5- to 6-fold (P < 0.05) with SPI+ or GEN serum, relative to CAS serum. PTEN expression was also higher in MCF-7 cells grown in the presence of SPI+ (P = 0.08) and GEN (P < 0.05) serum than in cells grown in CAS serum (Figure 6B). A similar trend in the induction of PTEN gene expression was observed when primary cultures of rat mammary epithelial cells were treated with serum from CAS, SPI+ and GEN-fed rats (Figure 6B). Analysis of total GEN concentrations in serum samples indicated levels of 1.47 ± 0.47 μM, 0.44 ± 0.10 μM and undetectable for rats on GEN, SPI+ and CAS diets, respectively (53).
incubated with CAS serum alone. Transfection with the irrelevant (negative control) siRNA had no effect on apoptosis for any of the treatments (data not shown).

Next, we determined whether disruption of PTEN expression resulting in attenuation of serum (GEN and SPI\textsuperscript{+})-induced apoptosis, altered the expression levels of the pro-apoptotic genes Bax, p21 and Bok in serum-treated MCF-7 cells. Consistent with their effects on apoptosis, serum from rats fed GEN increased the expression of the pro-apoptotic genes p21 (effect of GEN > SPI\textsuperscript{+}; \(P < 0.001\)) and Bok (effect of GEN = SPI\textsuperscript{+}), relative to CAS serum, albeit the expression levels of Bax did not similarly change with these treatments (Figure 8). Suppression of PTEN expression by PTEN siRNA resulted in diminished p21 expression, with greater effects noted in cells treated with GEN and SPI\textsuperscript{+} serum, than in those treated with CAS serum. Expression of Bok was also inhibited by blocking PTEN function under all treatments. By contrast, PTEN siRNA had no effect on Bax gene expression in cells treated with CAS and GEN serum, but increased levels of Bax transcripts in cells treated with SPI\textsuperscript{+} serum (Figure 8).

**Discussion**

The cellular and molecular mechanisms underlying the protective effects of soy-based diets on hormone-related cancers, including breast cancer remain unclear. Data from \textit{in vivo} and \textit{in vitro} studies have linked GEN, the primary soy-derived isoflavone to this cancer protection via its ability to induce target cell apoptosis (12,18), dysregulation of which is a hallmark of tumor cells and underlies tumor progression, metastasis and aggressiveness. In the present study, we demonstrated the apoptotic-inductive properties of dietary soy protein (SPI\textsuperscript{+}) and its associated isoflavone genistein (GEN), the latter when consumed as a dietary supplement, on mammary gland structures \textit{in vivo} and defined a novel signaling pathway by which GEN induces apoptosis for mammary tumor protection. We found that mammary epithelial cells, when exposed to SPI\textsuperscript{+} and GEN \textit{in vivo} or to sera derived from rats fed these diets \textit{in vitro} had increased PTEN expression, coincident with their increased apoptotic status. Suppression of PTEN expression \textit{in vitro} abolished apoptosis induced by GEN sera in the mammary epithelial cell line MCF-7, but did not totally inhibit that induced by SPI\textsuperscript{+} sera. Results suggest that while GEN predominantly utilizes the PTEN signaling pathway for induction of cellular apoptosis in mammary epithelial cells, additional pathways, possibly engaged by
other soy-associated components contribute to the apoptotic activity of dietary soy.

Here we have established the relevance of PTEN in the pro-apoptotic activity of GEN on mammary epithelial cells. We showed that the mammary structures LOB and DE from young adult (PND50) rats lifetime exposed to GEN diet had higher levels of the PTEN protein and concomitantly, increased apoptotic status, relative to those fed the control CAS diet.

We further showed that pure GEN as well as sera from rats lifetime exposed to GEN-containing diets induced PTEN expression and also apoptosis in MCF-7 cells, mimicking that observed in vivo. In addition, we demonstrated that induction of apoptosis in MCF-7 cells by GEN or GEN sera was abrogated by siRNA directed against PTEN. Finally, interference of PTEN expression in vitro resulted in inhibition of GEN sera-induced expression of the pro-apoptotic genes p21 and

Fig. 7. Apoptotic status of MCF-7 cells treated with serum (1% final concentration) from PND50 rats lifetime exposed to dietary CAS, SPI* or GEN, in the presence and absence of transfected PTEN siRNA (100 nM). (A) Representative fields showing apoptotic cells in mock-transfected (−siRNA) and PTEN siRNA-transfected (+siRNA) MCF-7 cells, treated with rat serum from the three diet groups. (B) The number of apoptotic cells for each diet group was counted from an average of 200–300 cells per field, with 3 fields evaluated per slide, and 3 slides evaluated per treatment group. Data are means ± SEM; P < 0.05 for means with different letters, when analyzed by two-way ANOVA. See online Supplementary material for a color version of this figure.
Bok, whose mammary expression in vivo are enhanced by dietary GEN. These results indicate that PTEN activation by GEN may be essential for apoptosis induction in specific mammary gland structures (LOB and DE) in young adult rats. The lack of a similar causal relationship between PTEN expression and apoptosis in TEB, the structures most sensitive to chemical carcinogenesis at the age examined here, is unclear at the present time and will require further studies. However, this may be related to the differentiation status of the TEBs, relative to LOBs and DE at PND50; the phosphorylation status of PTEN protein, which has been shown to inhibit its activity (54) in TEBs; and the fact that regulation of apoptosis is a complex, context-dependent process involving signaling pathways other than PTEN.

The mechanism(s) by which GEN induces PTEN expression leading to apoptosis is presently unclear. However, this is

**Fig. 8.** Effect of PTEN siRNA on apoptotic gene expression by MCF-7 cells treated with serum (1% final concentration) from PND50 rats lifetime exposed to dietary CAS, SPI or GEN. The expression levels of PTEN, p21, Bax and Bok were determined by qPCR. Data are means ± SEM from three independent experiments; P < 0.05 for means with different letters, when analyzed by two-way ANOVA.
probably unrelated to GEN’s function as a general inhibitor of tyrosine-specific protein kinases since the latter activity of GEN was shown to be effective only at high doses (100 μM) (16), while the coincident induction of PTEN expression and apoptosis in vivo and in vitro reported here occurred at >50-fold lower concentrations. Consistent with this, the inhibition of NMU-induced rat mammary tumorigenesis by GEN was reported to be independent of its inhibition of tyrosine kinase activity (55). Studies to delineate whether GEN modulates transcription of the PTEN gene are ongoing in our laboratory.

The recent report of GEN’s inactivation of the anti-apoptotic transcription factor NF-κB by inhibiting the phosphorylation of Akt (19) is consistent with the observed GEN induction of PTEN expression reported here. PTEN dephosphorylation of phosphatidylinositol 3,4,5-triphosphates leads to negative regulation of the PI3-K pathway, resulting in reduced Akt phosphorylation (36). Interestingly, the inhibition of NF-κB as well as Akt kinase activities in the earlier study was noted at 50 μM, in contrast to the much lower effective in vivo concentration for induction of apoptosis and PTEN expression reported here, based on the serum levels of GEN found in rats fed these diets (SPI+, 0.44 μM; GEN, 1.47 μM). Further studies to correlate the temporal and dose-dependent effects of GEN on PTEN induction and Akt inactivation are needed to address this apparent discrepancy. Interestingly, NF-κB has been demonstrated to inhibit PTEN gene expression (56); thus, PTEN, Akt, and NF-κB probably constitute a tightly regulated loop for the maintenance of cell apoptosis and homeostasis, and which is influenced by nutrition.

We have determined that PND50 rats lifetime exposure to dietary GEN and SPI+ differed in their mean serum genistein concentrations, despite comparable amounts of GEN aglycone equivalents in these diets (216 versus 250 mg/kg SPI+ and GEN diet, respectively). This apparent discrepancy is probably related to the molecular forms of dietary GEN in SPI+ (glucoronide and sulfate conjugates; free and protein-bound aglycones) versus the supplement (free aglycone) (57). There is also the possibility that absorption and processing of soy protein-associated GEN is significantly different from pure GEN. Nevertheless, since the serum levels of GEN determined for rats on SPI+ diet are similar to those previously reported for humans regularly consuming soy products (0.3–0.6 μM) (58), the observed induction of mammary epithelial cell apoptosis by soy demonstrated in female rats in the present study may have biological relevance in human females.

It is worth noting that while serum from rats fed GEN and SPI+ induced comparable responses in gene expression and apoptosis in MCF-7 cells, mammary glands from GEN-fed rats had greater apoptosis and enhanced expression of the pro-apoptotic genes p21, Bax and Bok, relative to those fed SPI+. Several possibilities may explain these observations. First, other soy-associated components may negate and/or modify the apoptotic activity of GEN present in soy. Second, mammary stromal cells in vivo may serve as targets for and/or modify the epithelial cell actions of, other soy-associated bioactive components, resulting in altered stromal/epithelial cell interactions that are important for in vivo epithelial cell response. Last, PTEN expression in the stromal compartment, mutations of which have been reported in breast carcinoma (32) may also mediate the apoptotic response of mammary gland to GEN; however, we did not observe significant differences in stromal PTEN expression as a function of diet (data not shown). Although further experiments are required to evaluate these possibilities, our observations of the distinct biological effects of GEN in vivo when taken as a dietary supplement versus when consumed as a normal constituent of soy foods, raise questions on how the signaling pathways induced by nutritional factors present in foods may collaborate for optimal health benefits.

The present findings provide the groundwork for addressing two important questions related to diet and adult risk of mammary cancer. One relates to the underlying mechanism for the pro-apoptotic effects of dietary SPI+ and GEN in vivo, which do not appear to be dependent solely on circulating GEN levels. We have found that SPI+ and GEN serum exhibit comparable apoptotic activities, despite their distinct levels of GEN (3-fold higher in GEN than in SPI+). Moreover, the apoptotic activities of GEN and SPI+ sera in vitro (with GEN levels in the range of 4–15 nM, when serum was added at 1% final concentration) were higher than that of pure GEN used in the μM range. Given that at least 15% of all cancers are now considered to be caused by inflammation (59,60); that cytokines are powerful modulators of the immune system (61), and that NF-κB, an inflammation-induced transcription factor central to the inflammatory response, is downregulated by GEN (20), possibly through PTEN inactivation of Akt, an analysis of the differential expression of NF-κB-regulated pro-inflammatory and immunosuppressive cytokines in serum from SPI+ and GEN rats, relative to those of CAS rats, may provide important insights into the mechanisms of GEN action distinct from those of SPI+. More importantly, such findings may suggest dietary manipulation of the immune system as a viable alternative for cancer protection (62).

The other question relates to the nature/lineage of the mammary cell populations in vivo that are highly sensitive to apoptosis induced by dietary intake of SPI+ and GEN. It has been suggested that a population of undifferentiated cells with high tumorigenic potential is present in the developing mammary gland, and that removal of these cells at an early developmental period could lead to mammary phenotypes that are less susceptible to genetic mutations at a later life stage (63,64). It is tempting to speculate that the few numbers of cells induced to undergo apoptosis by dietary SPI+ and GEN represent progenitor cells that are targets for transformation during mammary tumorigenesis, and that their precise targeting may be related to increased PTEN expression. The application of epithelial progenitor cell markers such as keratin 6 and Sca-1 (65) to identify the cell populations targeted for apoptosis by dietary SPI+ and GEN should enable examination of this possibility.

In summary, we have presented evidence of a role for PTEN in the induction of mammary epithelial cell apoptosis by GEN. We further show that the signaling pathway for activation of apoptosis by dietary SPI+ may be a manifestation of the cumulative effects of other soy components, in addition to PTEN-mediated GEN action. Our findings provide a framework for functional dissection of the involvement of PTEN in mammary tumor protection as mediated by dietary factors, and present new insights concerning the regulation of systemic factors by diet that could lead to new approaches for cancer treatment and prevention.

Supplementary material

Supplementary material is available online at: http://carcin. oxfordjournals.org/.


*Received February 20, 2005; revised April 11, 2005; accepted May 13, 2005*