Repression of mammosphere formation of human breast cancer cells by soy isoflavone genistein and blueberry polyphenolic acids suggests diet-mediated targeting of cancer stem-like/progenitor cells

Maria Theresa E. Montales1,2; Omar M. Rahal1,3; Jie Kang1,2; Theodore J. Rogers4; Ronald L. Prior4; Xiali Wu5,6; Rosalia C. M. Simmen1,2,3,*

1Arkansas Children’s Nutrition Center, 15 Children’s Way, 2Department of Physiology and Biophysics and 3Interdisciplinary Biomedical Sciences Program, University of Arkansas for Medical Sciences, Little Rock, AR 72202, USA
*To whom correspondence should be addressed. Tel: +1 364 2849; Fax: +1 364 3161; E-mail: simmenrosalia@uams.edu

Mammary stem cells are undifferentiated epithelial cells, which initiate mammary tumors and render them resistant to anticancer therapies, when deregulated. Diets rich in fruits and vegetables are implicated in breast cancer risk reduction, yet underlying mechanisms are poorly understood. Here, we addressed whether dietary factors selectively target mammary epithelial cells that display stem-like/progenitor subpopulations with previously recognized tumor-initiating potential. Using estrogen receptor-positive MCF-7 and estrogen receptor-negative MDA-MB-231 human breast cancer cell lines and freshly isolated epithelial cells from MMTV-Wnt-1 transgenic mouse mammary tumors, we demonstrate that sera of adult mice consuming soy isoflavone genistein (GEN) or blueberry (BB) polyphenol-containing diets alter the population of stem-like/progenitor cells, as measured by their functional ability to self-renew and form anchorage-independent spheroid cultures in vitro at low frequency (1–2%). Serum effects on mammosphere formation were dose-dependently replicated by GEN (40 nM > 2 μM) and targeted the basal stem-like CD44+/CD24−/ESA+ and the luminal progenitor CD24+ subpopulations in MDA-MB-231 and MCF-7 cells. GEN inhibition of mammosphere formation was mimicked by the Akt inhibitor perifosine and was associated with enhanced tumor suppressor phosphatase and tensin homologue deleted on chromosome ten (PTEN) expression. In contrast, a selected mixture of BB phenolic acids was only active in MDA-MD-231 cells and its CD44−/CD24−/ESA+ subpopulation, and this activity was independent of induction of PTEN expression. These findings delineate a novel and selective function of distinct dietary factors in targeting stem/progenitor cell populations in estrogen receptor-dependent and -independent breast cancers.

Introduction

Breast cancer, like many other human cancers, is considered to be derived from and maintained by a small population of self-renewing tumor-initiating cells, designated as cancer stem cells (CSCs) (1–5). Nonetheless, the origin of CSC remains to be fully elucidated. The long-held notion is that of a unidirectional hierarchical model wherein a pool of stem cells (SC) residing at the top of the epithelial hierarchy undergoes oncogenic transformations, selectively endowing these cells with tumor-initiating properties while maintaining their ability to generate more differentiated progeny lacking tumorigenic potential (1,6). In a recent study, Weinberg et al. presented strong evidence for the bidirectional interconversion of CSC and non-CSC in human mammary epithelial cell populations (7), thus expanding if not replacing the latter model. Although it is thought that CSC and normal SC share developmental programs that regulate critical SC fate and maintenance (8), the control of growth and differentiation-associated pathways in cells-of-origin of CSC likely diverged or is subverted from those of normal counterparts (9). In this regard, deregulation of Wnt, Notch, Hedgehog and phosphatase and tensin homologue deleted on chromosome ten (PTEN)/phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways, all of which constitute growth-control pathways for normal SC have been shown to underlie aberrant CSC self-renewal leading to breast cancer (10–13).

The incidence of breast cancer varies worldwide, a consequence in part of environmental rather than genetic differences and implicating dietary and lifestyle disparities among the general population (14). Prevailing evidence from epidemiological and experimental data suggest that breast cancer development can be influenced by diet/nutrition (15–18). Previous studies by our group (19,20) and others (21) have shown that dietary intake of soy-rich foods containing genistein (GEN) and GEN-supplemented diets inhibit chemically-induced mammary tumor formation in rodent models, in part through inhibition of Wnt signaling and upregulation of PTEN expression (22–25). Likewise, we have shown that in rodent models, early exposure to blueberry (BB) solely through maternal diet, enhanced mammary epithelial differentiation in prepubertal progeny, a process mediated by upregulation of PTEN expression and its nuclear localization (26). Given that PTEN and Wnt signaling pathways are functionally linked (27) and that these same pathways constitute key regulators of mammary epithelial SC biology (1,8,11,12), our findings raise the interesting possibility that SC, specifically CSC comprise viable targets of dietary factors.

Breast cancer cell lines, MCF-7 and MDA-MB-231, have been shown to display a subpopulation of cells with SC-like properties, defined experimentally by their ability to grow as spheroids in the absence of attachment and to self-renew in secondary or additional passages in vitro (3,5,28). These cell lines contain an ALDEFLUOR-positive population expressing the SC marker aldehyde dehydrogenase (29), which was previously used to isolate epithelial subpopulations displaying CSC properties from human breast tissues and breast carcinomas (30). Furthermore, flow cytometric analyses of MDA-MB-231 cells characterized a subpopulation displaying the CD44+/CD24−/ESA− phenotype, which when grafted to non-obese diabetic/severe combined immunodeficient mice at limiting dilution, rapidly formed tumors (28). The availability of these in vitro models in which CSC reside in a niche with more differentiated progeny approximating that in vivo, provide opportunities for addressing proof-of-concept questions of potential clinical significance for mitigating breast cancer relapse and drug resistance (1–4,31).

In the present study, we utilized MCF-7 and MDA-MB-231 cells to address the postulate that mammary stem/progenitor cells with tumor-initiating potential are direct targets of dietary components with known anti-breast cancer effects. MCF-7 is a well-differentiated estrogen receptor (ER)-positive breast cancer cell line, whereas MDA-MD-231 is a highly metastatic ER-negative cell line (32). To provide evidence for a role for dietary factors in limiting SC/progenitor cell-enriched populations, we evaluated sera from adult female mice consuming isocaloric and isonitrogenous diets containing GEN and BB, pure GEN as well as a select mixture of BB polyphenolic acids, for their ability to inhibit mammosphere formation in both cell lines. We show here that the inhibitory activity of sera from GEN-exposed animals was recapitulated by exogenous GEN in both cell lines and in freshly isolated epithelial cells from Wnt-1 transgenic (Wnt-Tg) mice at limiting dilution, rapidly formed tumors (28).

Abbreviations: BB, blueberry; CAS, casein; CSC, cancer stem cell; ER, estrogen receptor; GEN, genistein; PI3K, phosphatidylinositol 3-kinase; PR, progesterone receptor; PTEN, phosphatase and tensin homologue deleted on chromosome ten; SC, stem cell; Wnt-Tg, Wnt-1 transgenic.

© The Author 2012. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com
mouse mammary tumors (33). Furthermore, we show that GEN inhibition of mammosphere formation was associated with attenuated PI3K/Akt signaling and upregulated PTEN expression and targeted the CD44+/CD24-/ESA+ subpopulation, previously shown to generate tumors in immunocompetent mice (28) as well as the luminal progenitor-enriched CD24+ cells. In contrast, a select mixture of phenolic acids found in sera of BB-exposed animals inhibited mammosphere formation only in the more aggressive MDA-MB-231 cells and its CD44+/CD24-/ESA+ subpopulation. Our findings indicate SC/progenitor cells as functional targets of dietary factors with breast cancer risk reduction activities and expand the repertoire of diet-mediated PTEN-associated pathways that may be exploited to avert the occurrence and relapse of ER-positive and ER-negative breast cancers.

### Materials and methods

#### Animal studies

Animal studies were carried out under protocols approved by the Institutional Animal Care and Use Committee, University of Arkansas for Medical Sciences. Mice were housed in polycarbonate cages under conditions of 24°C, 40% humidity and a 12 h light–dark cycle. MMTV-Wnt-Tg mice (33) were obtained from Jackson Laboratory (Bar Harbor, ME). Wild-type female mice of the same strain and Wnt-Tg males were mated to generate wild-type and Wnt-Tg female offspring. Genotyping protocols were described previously (27). Adult female mammary gland epithelial cells (3-6 months) were lifetime exposed (beginning at gestation day 4) to one of three semipurified isocaloric diets made according to the American Institute of Nutrition-93G formulation (19) and served as source of sera for in vitro treatments (described below). These diets are: (i) Casein (C), (ii) Casein (C), (iii) BB, CAS supplemented with $300$ mg/kg food; and (iv) BB, CAS supplemented with $300$ mg/kg food. CD3 and 25 mM potassium nitrate (GIBCO). Cells were incubated in 5% CO2:95% air at 37°C and 2 mM glutamine (GIBCO). The MDA-MB-231 culture medium contained 10% fetal bovine serum (GIBCO, Carlsbad, CA) and 1% antibiotic–antimycotic solution (GIBCO). The MDA-MB-231 and MCF-7 cell lines were propagated in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (GIBCO, Carlsbad, CA) and 1% antibiotic–antimycotic solution (GIBCO). The MDA-MB-231 culture medium contained 10% fetal bovine serum (GIBCO, Carlsbad, CA) and 1% antibiotic–antimycotic solution (GIBCO). The MDA-MB-231 culture medium contained 10% fetal bovine serum (GIBCO, Carlsbad, CA) and 1% antibiotic–antimycotic solution (GIBCO). The MDA-MB-231 culture medium contained 10% fetal bovine serum (GIBCO, Carlsbad, CA) and 1% antibiotic–antimycotic solution (GIBCO). The MDA-MB-231 culture medium contained 10% fetal bovine serum (GIBCO, Carlsbad, CA) and 1% antibiotic–antimycotic solution (GIBCO). The MDA-MB-231 culture medium contained 10% fetal bovine serum (GIBCO, Carlsbad, CA) and 1% antibiotic–antimycotic solution (GIBCO). The MDA-MB-231 culture medium contained 10% fetal bovine serum (GIBCO, Carlsbad, CA) and 1% antibiotic–antimycotic solution (GIBCO).

#### Mammosphere formation assay

MCF-7 and MDA-MB-231 cells, when seeded in ultra-low attachment plates (Corning, Cornyn, NY) in serum-free media form non-adherent spheroids termed mammospheres, with the ability to self-renew (28). Plating medium for mammosphere formation consisted of phenol red-free serum-free Minimal essential media supplemented with B27 (1; Invitrogen), 20 ng/ml basic fibroblast growth factor (In Vitro), 20 ng/ml human epidermal growth factor (In Vitro), 10 ng/ml heparin (Sigma–Aldrich), 1% antibiotic–antimycotic solution (Invitrogen) and 100 ng/ml gentamicin (Sigma–Aldrich). To examine the effects of various treatments on mammosphere formation, MCF-7 and MDA-MB-231 cells were seeded in 24-well ultra-low attachment plates in plating medium with and without added treatments at a density of 2500 cells per well. Plating medium was refreshed every 3 days in the absence of additional treatments and the appearance of primary spheres (P1) was evaluated after 5 days. Mammospheres with diameters of $1000$ µm (MCF-7) and $60$ µm (MDA-MB-231) were manually counted using a Carl Zeiss Axiovision microscope (Carl Zeiss AG, Oberkochen, Germany). To assess the relative sphere numbers over second (P2) and third (P3) passages, mammospheres from the previous plating were collected at day 5 (P1) or 7 (P2), dissociated with 0.05% trypsin (Invitrogen) into single-cell suspensions, filtered using a 40 µm sieve and replated in ultra-low attachment plates, with no additional treatments. Treatment effects were determined from at least three independent experiments in quadruplicates.

### Suppression of mammosphere formation by GEN and BB phenolic acids

#### Mammary tumor epithelial cells, treatments and mammosphere formation assay

The isolation of mouse mammary tumor epithelial cells followed previously described protocols (34,35). Briefly, mammary tumors removed from Wnt-Tg females were incubated in digestion medium (Dulbecco's modified Eagle medium/F12 containing 100 µg/ml gentamicin, 1% antibiotic–antimycotic and Collagenase Type III (225 units/ml/g tissue; Worthington, Lakewood, NJ)) at 37°C for 2.5 h in a rotary shaker at 125 r.p.m. Cells were filtered through 40 µm cell strainers, washed four times in washing buffer (Dulbecco's modified Eagle medium/F12, 5% fetal bovine serum and 50 µg/ml gentamicin) at 2000 r.p.m. for 2 s and then in phosphate-buffered saline. Subsequent evaluation of mammosphere numbers followed that described for the breast cancer cell lines (above), except that cells were plated in six well-attachment plates at a density of 10 000 (P1) and 5000 (P2) cells per well.

#### RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was prepared from mammospheres collected at days 5 (P1) and 7 (P2) post-plating, using Trizol reagent (Invitrogen), subjected to RNase-free DNase treatment and converted into complementary DNA by using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time polymerase chain reaction analyses used SYBR Green and the ABI Prism 7000 Detection System (Applied Biosystems, Foster City, CA), and primer design was performed as described (25). The primers used for gene expression analyses are presented in Supplementary Table 1, available at Carcinogenesis Online. TATA-box binding protein messenger RNA was used as normalizing RNA and fold-change was calculated based on vehicle-treated normalized values for each transcript.

#### Fluorescence-activated cell sorting

The phenotypes of parental MCF-7 and MDA-MB-231 cells grown in plastic were determined by flow cytometry using a FACS Aria cell sorting flow cytometer (BD Biosciences) and human luminal (CD24+PE) and basal (CD44+/CD24-/ESA+) epithelial markers (BD Pharmingen, San Jose, CA). MDA-MB-231 cells were enriched for tumor-initiating cells by flow cytometry using CD44-APC, CD24-PE (both from BD Pharmingen) and ESA-fluorescin isothiocyanate (Stem Cell Technologies, Vancouver, Canada). Briefly, cells (1 $10^6$/ml) were resuspended in Hank's balanced salt solution containing 2% fetal bovine serum and $100$ mM $N$-2-hydroxyethylpiperazine-$N$-2-ethanesulfonic acid and stained with primary antibodies (1:100 dilution) or isotype controls for 15 min at room temperature. Cells with the CD44+/CD24+/ESA+ phenotype were used for mammosphere formation at a seeding density of 10 000 (P1) and 5000 (P2) cells per well. P1 and P2 mammospheres were collected for gene expression analyses by quantitative real-time polymerase chain reaction.

#### Analyses of serum metabolites

Sera collected from adult female mice assigned to CAS- or BB-diets (n = 6 per dietary group) were analyzed for phenolic metabolites following previously published procedures (36).

#### Data analysis

The statistical significance of differences in numerical data was evaluated using SigmaStat version 3.5 for Windows. Data were analyzed using Student’s t-test or one-way analysis of variance. A P value <0.05 was considered to be statistically significant.

### Results

#### Mammosphere formation by MCF-7 and MDA-MB-231 cells

We initially characterized the size of the basal and luminal subpopulations within MCF-7 and MDA-MB-231 cell lines using specific antigens (CD44+ for basal; CD24+ for luminal). Consistent with a previous study (28), MCF-7 cells are highly enriched for the CD24+ subpopulation, whereas MDA-MB-231 cells are predominantly CD44+, with low to nil expression of CD24 (Figure 1a and b). A small population of human breast cancer cells can survive and proliferate in vitro as floating spherical colonies under anchorage-independent conditions (5). These spheres, designated mammospheres given their mammary epithelial origin, exhibit SC-like/progenitor properties based on their ability to self-renew and initiate and/or sustain heterogeneous tumors. To confirm if the luminal epithelial-enriched, ER/progesterone receptor (PR)-positive MCF-7 and basal epithelial-enriched, ER/PR-negative MDA-MB-231 cells contain the SC/progenitor population as reported previously (28), cells were seeded in ultra-low attachment plates and spheres formed from the...
original plating (P1) were collected and examined for subsequent formation of mammospheres after replating (P2 and P3). Both cell lines showed a small subset (1–2%) of the epithelial population capable of forming mammospheres upon initial plating (P1; Figure 2a and b).

Mammospheres formed from MCF-7 cells tended to be bigger (≥100 μm on average) than those of MDA-MB-231 cells (~60 μm on average). The percent of mammospheres formed was significantly increased from P1 to P2 for each cell line, suggesting enrichment of a subpopulation with self-renewal capacity. However at P3, mammosphere formation was either maintained at the P2 level (MCF-7) or reduced to that of P1 (MDA-MB-231); this is consistent with decreased self-renewal capacity of luminal progenitor cells with serial passage (37). Thus, we considered P1 and P2 mammospheres of both cell lines to contain a mixture of basal stem and luminal progenitor cells; further analyses were conducted with these cell passages.

Mammosphere formation by MCF-7 and MDA-MB-231 cells is inhibited by sera from mice consuming dietary GEN and BB. Diet and dietary factors modify breast cancer risk as shown in animal models and epidemiological studies (16–21). To investigate whether dietary factors can potentially target breast cancer cells with stem/progenitor properties in vivo, we conducted ex vivo studies wherein sera pools from adult female mice (n = 6 per group) fed CAS, GEN and BB were added at 1 and 5% (vol/vol) final concentrations (in mammosphere plating medium) to MCF-7 and MDA-MB-231 cells upon seeding in ultra-low attachment plates. Mammosphere formation at P1 was quantified 5 days later. Although MDA-MB-231 cells had a 10-fold lower efficiency than MCF-7 cells to generate mammospheres in suspension culture, sphere formation in both cell lines was significantly decreased by GEN-sera and BB-sera relative to CAS-sera at the

Fig. 1. Distinct epithelial phenotypes of human MCF-7 and MDA-MB-231 breast cancer cell lines. (a) Representative fluorescence-activated cell sorting analysis of MCF-7 and MDA-MB-231 cells using specific surface antigens for basal (CD44-APC) and luminal (CD24-PE) epithelial cells. In all experiments, cells were gated with isotype controls, following previously published protocols (28). (b) Summary of the percentage of cells positive for CD24 and CD44 for each cell line from n = 3 independent experiments.

Fig. 2. Human breast cancer lines form mammospheres that are sensitive to factors found in sera of mice consuming various diets. (a) MCF-7 and (b) MDA-MB-231 cell lines were seeded to form primary mammospheres (P1) at a density of 2500 cells per well in 24-well low-attachment plates. After 5 days, P1 mammospheres were counted, collected and replated under the same conditions at a density of 1000 cells per well to form secondary mammospheres (P2). Data for P3 were obtained from P2. Mammospheres formed (% mean ± SEM) are expressed relative to number of cells plated for each passage and are from three independent experiments. Mean values with different letter subscripts differed at P < 0.05. Inset: Primary mammospheres formed from unsorted MCF-7 and MDA-MB-231 cells in suspension culture for 5 days. Magnification = ×100, scale bar = 50 μm. (c and d) Addition of sera from mice exposed to GEN- and BB-diets at 5 and 1% (v/v) final concentrations to plating medium, significantly reduced mammosphere formation of MCF-7 and MDA-MB-231 relative to control CAS diet. P1 spheres were counted at day 5 after plating. Data represent the number of mammospheres per 2500 plated cells (mean ± SEM) for each serum concentration. Values with different letters (a, b) differed at P < 0.05. N = 3 independent experiments, with each experiment carried out in quadruplicates.
Soy isoflavone GEN attenuates mammosphere formation by breast cancer cell lines and mammary tumor epithelial cells

GEN is bioavailable in humans and rodents consuming soy foods (17,18,38,39) and may underlie the decreased breast cancer risk attributed to dietary soy intake. To examine if GEN treatment limits mammosphere-forming ability, MCF-7 and MDA-MB-231 cells were treated at plating with GEN within the concentration range (40 nM and 2 μM) found in sera of regular soy food consumers (17,38,39) and evaluated for numbers of mammospheres formed at P1 and P2. GEN attenuated mammosphere formation in both cell lines at P1 and P2, with the lower dose (40 nM) eliciting consistently greater inhibitory effects than the higher (2 μM) dose (Figure 3a and b). Interestingly, although GEN at 2 μM dose was only effective in P2 mammospheres generated from MCF-7 cells, MDA-MB-231 cells were comparably insensitive to 2 μM GEN at P1 and P2.

Only a small subset of MCF-7 and MDA-MB-231 cells, specifically those characterized by the CD44+/CD24-/ESA+ phenotype, exhibits bona fide CSC-like properties based on xenograft transplantation (28). To address whether GEN specifically target this subpopulation, MDA-MB-231 cells were isolated by fluorescence-activated cell sorting using fluorophore-labeled antibodies to CD44, CD24 and ESA; this isolation procedure resulted in a 2.5–5% yield of the CD44+/CD24-/ESA+ subpopulation (data not shown). These cells were seeded at low density (P1, 2500 cells per well; P2, 1000 cells per well) in ultra-low attachment plates and examined for GEN effects on mammosphere formation.

GEN inhibition of mammosphere formation involves PI3K/Akt signaling

Studies suggest that the PTEN/PI3K/Akt signaling pathway is involved in tissue-specific SC self-renewal (11). To evaluate whether GEN inhibits expansion of cells with self-renewal capabilities via this pathway, MCF-7 cells were treated with the Akt inhibitor perifosine (Akt-I, 0.5 μM) and GEN (40 nM), alone and together, at plating. Addition of Akt-I dramatically suppressed (by 70%) primary mammosphere formation in MCF-7 cells; this effect was effectively mimicked by 40 nM GEN (Figure 4a). Cells cotreated with 40 nM GEN + 0.5 μM Akt-I did not differ in mammosphere formation activity from those of cells treated with either GEN or Akt-I alone.

Fig. 3. GEN decreases the number of mammosphere-forming units (MFUs) in human breast cancer cell lines and in freshly isolated epithelial cells from mouse mammary tumors. (a) MCF-7 and (b) MDA-MB-231 cells were treated with GEN (40 nM and 2 μM) added to plating medium only at initial plating. Primary (P1) and secondary (P2) mammospheres were counted at 5 and 7 days, respectively after plating. Values are mean MFU ± SEM, normalized to those of control (vehicle only)-treated cells from three independent experiments. (c) GEN (40 nM and 2 μM) added to plating medium only at initial plating diminished the numbers of P1 and P2 mammospheres formed from CD44+/CD24-/ESA+ subpopulation isolated from MDA-MB-231 cells, relative to control (vehicle only)-treated cells. (d) Freshly isolated epithelial cells from mammary tumors of Wnt-Tg mice were treated with GEN (40 nM and 2 μM) added to plating medium only at initial plating. P1 mammospheres were collected and passaged for P2 without additional GEN treatment. Data (mean MFU ± SEM) are from P2 mammospheres of two independent experiments. Each experiment utilized mammary tumors from different mice. Means with different letters (a, b) differed at P < 0.05.
PTEN inhibition of mammosphere formation by mammary epithelial cells is associated with PTEN/PI3K/Akt regulatory pathway. (a) MCF-7 cells treated with Akt inhibitor perifosine (Akt-I) and GEN (40 nM), alone and together or vehicle alone, were evaluated for numbers of P1 mammospheres formed. Data are mean MFU ± SEM, relative to those of vehicle-only treated cells (n = 3 independent experiments). Means with different letters (a,b) differed at P < 0.05. (b) GEN induction of PTEN expression (quantified by quantitative real-time polymerase chain reaction) in P1 and P2 mammospheres is dose-dependent, with 40 nM showing a greater effect than 2 μM. Means with different letters (a, b) differed at P < 0.05 (n = 3 independent experiments), relative to vehicle-treated cells. (c) Transcript levels of cyclin D1 (CD1), ERα, GATA3 and PR in P1 mammospheres of MCF-7 cells treated with GEN (40 nM and 2 μM in 1% dimethyl sulfoxide) or vehicle alone (1% dimethyl sulfoxide). Transcript levels were quantified by quantitative real-time polymerase chain reaction and normalized to TATA-box binding protein and then renormalized to control (vehicle) values. Means with different letters (a, b) differed at P < 0.05. (d) Transcript levels of PTEN in P2 mammospheres formed from CD44+/CD24⁻/ESA⁺ subpopulation of MDA-MB-231 cells were increased by GEN treatment. Means with different letters (a,b) differed at P < 0.05, relative to vehicle-treated cells (n = 2 independent samples).

BB phenolic acid mixture selectively inhibits mammosphere formation by MDA-MB-231 cells

Given our findings (Figure 2c and d) that sera from mice consuming dietary BB inhibited mammosphere formation in MCF-7 and MDA-MB-231 cell lines, we evaluated the effects of BB phenolic acids on mammosphere formation in vitro. We utilized an artificial mixture of the seven phenolic acids, namely hippuric acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxybenzoic acid, 3-(3,4-dihydroxyphenyl)-propionic acid, ferrulic acid, 3-(4-hydroxyphenyl)-propionic acid and 3-hydroxybenzoic acid, based on a previous analyses of these compounds in sera of rats fed on 10% BB-diets (36). A range of concentrations (designated 5 ×, 10 × and 20 ×) higher than those found in sera of rats fed on BB diet was used for the treatments to take into account the potential for under-estimation of the bioactive phenolic acid concentrations in vivo due to their ability to be metabolized via conjugation with glucuronides or sulfates and which may render them inactive (41). Addition of the phenolic acid mixture to MDA-MB-231 cells suppressed primary mammosphere formation (P1) in a dose-dependent manner, with the lower doses (5 × and 10 ×) showing greater inhibitory effects than the highest dose (20 ×), which had no activity relative to vehicle control (Figure 5a). The inhibitory effects of the mixture at the two lowest concentrations (5 × and 10 ×) were sustained in P2 mammospheres. Interestingly, the highest dose (20 ×) showed comparable inhibitory activity as the lower doses on mammosphere formation upon secondary passage.

Hippuric acid is the most abundant phenolic acid in sera of rats exposed to dietary BB (36). We measured its concentration in sera of mice exposed to BB-diets and found this to be 2.84 ± 0.33 μg/ml (n = 3 independent samples), which was ~36-fold higher than in sera of mice exposed to dietary BB. Analyses of BB-sera for the other six phenolic acids present in the artificial mixture showed levels that were considerably lower than those found for hippuric acid and did not significantly differ from those found in...
We then evaluated the effect of hippuric acid on mammosphere formation at 3 μg/ml and 10 μg/ml, which represent physiological and supra-physiological doses, respectively. Although a 50% decrease in primary mammosphere formation was shown with the lower dose of hippuric acid, the higher dose was less effective and showed no significant inhibitory activity relative to control (Figure 5b).

Similar to that shown with GEN treatment (Figure 3c), mammosphere formation by CD44+/CD24-/ESA+ subpopulation of MDA-MB-231 cells was diminished (by 50%) with the addition of the BB phenolic acid mixture (5x dose). However, mammosphere formation by MCF-7 cells was not affected by the mixture at all doses examined (data not shown), despite the demonstrated effects of BB-sera on these cells (Figure 2c). Furthermore, the mixture at 5x concentration had no effect on mammosphere formation by epithelial cells derived from mammary tumors of Wnt-Tg mice (data not shown). Unlike that of GEN, the inhibitory effects of BB phenolic mixture and hippuric acid on mammosphere formation by MDA-MB-231 cells were not accompanied by increased PTEN expression (Figure 5d and e). Co-addition of BB phenolic mixture (5x) and GEN demonstrated the mammosphere-inhibitory activity of 40 nM GEN and the lack of effect of 2 μM GEN, respectively with (Figure 5f) and without (Figure 2c) added 5x BB in MCF-7 cells. The same cotreatments in MDA-MB-231 cells resulted in additive effects of GEN (40 nM) and 5x BB in inhibiting

657
mammosphere formation (data not shown), confirming the selective activity of BB phenolic mixture on MDA-MB-231 cells.

Discussion

In this report, we provide evidence indicating that bioactive factors in foods with putative antimammary tumor activities target a subpopulation of mammary epithelial cells with mammosphere-forming capacity characteristic of SC/progenitor cells. Our studies utilized two human breast cancer cell lines (MCF-7 and MDA-MB-231) with distinct cancer subtypes (28), a subpopulation of MDA-MB-231 cells (CD44+/CD24⁻/ESA⁺) with previously characterized tumor-initiating properties (28) and primary epithelial cells isolated from mammary tumors of a well-accepted mouse model of mammary tumorigenesis (MMTV-Wnt-1; Wnt-Tg) that recapitulates salient features of the human disease (33,34). We showed that GEN, a major soy isoflavone, and hippuric acid, a metabolite of BB polyphenols, can effectively attenuate mammosphere formation in vitro at physiologically relevant doses, reflecting their potential inhibitory effects on CSC self-renewal and expansion in vivo. We further showed that inhibition of PI3K/Akt signaling, associated with upregulation of PTEN expression constitutes a relevant pathway by which CSC/progenitor cell behavior may be controlled by particular dietary factors. Together, our results suggest a novel mechanism by which progression of breast cancer in women may be attenuated by consumption of ‘healthy’ diets. Given that cancer cells with SC-like properties underlie resistance of breast tumors to radiation and chemotherapeutic agents and hence increased tumor recurrence (2,4,5,31), our findings may have important implications for the design of dietary interventions for improving breast cancer outcomes.

The human breast cancer cell lines used in the present study (MCF-7 and MDA-MB-231) have been previously demonstrated to exhibit expression of the well-accepted SC marker aldehyde dehydrogenase expression, as assessed by the ALDEFLUOR assay (29, 30). Although we did not utilize this marker for isolating the epithelial subpopulation for mammosphere formation assays, the robust expression of CD44+/CD24⁻/ESA⁺ by the highly metastatic MDA-MB-231 and their corresponding lower expression in MCF-7 cells, as confirmed here, are consistent with the epithelial to mesenchymal transition traits associated with SC phenotype in ALDEFLUOR-positive cells (42). Furthermore, whereas freshly isolated mammary epithelial cells from Wnt-Tg tumors were not additionally fractionated for the CD29+/CD24⁻ subpopulation that was previously shown to display outgrowth potential using transplantation assay and hence SC properties (34), we found that these primary cells also exhibited reduced potential to self-renew in anchorage-independent conditions with low GEN dose (40 nM). This suggests the presence of an SC subpopulation in Wnt-Tg tumors that are responsive to GEN inhibitory activity. The collective findings establish MCF-7 and MDA-MB-231 cell lines as relevant in vitro models for large-scale screening of dietary and other factors for potential SC targeting activity to inform breast cancer therapy.

In previous studies (22,25), we showed that induction of PTEN expression and its increased nuclear localization are hallmarks of isoflavone GEN action in both non-tumorigenic mammary (e.g. MCF-10A) and breast cancer (MCF-7) cell lines. Increased PTEN expression in these cells resulted in decreased proliferative and increased apoptotic status, consistent with altered expression of genes involved in cell cycle arrest, survival and differentiation (22–25,43). The present studies suggest that GEN may act as a potent inhibitor of the expansion of cancer cells with SC/progenitor characteristics through its induction of PTEN expression in mammospheres. Although further studies will be required to mechanistically establish the functional association between GEN induction of PTEN expression and its ability to mimic the mammosphere-inhibitory activity of the Akt inhibitor perifosine (e.g. by PTEN small interfering RNA-mediated knockdown and measurement of activated (phosphorylated) AKT protein levels), these findings have important implications given that the PTEN/PI3K/Akt pathway is an important driver of the regulation of both normal and malignant mammary SC/progenitor cell numbers (11,34). The low frequency of mammospheres precluded our confirmation of the coincident increase in PTEN protein levels with transcripts, however, we have shown previously the coordinate induction of PTEN messenger RNA and protein by GEN treatment in mammary epithelial cells (22,25).

Bioactive concentrations of GEN at the nanomolar range are physiologically relevant; indeed, although a single oral GEN dose of 460 mg administered to humans resulted in peak plasma levels of 20 μM GEN, the levels that reached target tissues were significantly lower and within the nanomolar range (38,39). Based on the latter, we used 40 nM and 2 μM GEN in the present study. We observed that the lower GEN dose (40 nM) consistently attenuated primary and secondary mammosphere formation of transformed cell lines and primary epithelial cells isolated from mammary tumors; in contrast, the supra-physiological GEN dose (2 μM) was less effective in eliciting a similar biological outcome. For the MDA-MB-231 CD44+/CD24⁻/ESA⁺ subpopulation, which is highly enriched for CSC, however, both doses of GEN were equally effective in suppressing mammosphere formation. Furthermore, although the lower GEN dose highly induced PTEN transcript levels in mammospheres formed from the more differentiated MCF-7 cells expressing PTEN (22), the higher GEN dose displayed a more robust induction of PTEN expression in the CD44+/CD24⁻/ESA⁺ subpopulation of MDA-MB-231 cells. An experimental explanation for the noted distinct dose-dependent responses is lacking at the present time, however, these may be partly related to the reported pro-proliferative activity of high GEN concentrations (micromolar range) in mammary epithelial cells (44); and possibly, the higher sensitivity of the more differentiated luminal progenitor cells for PTEN induction by GEN. The robust inhibition of ERα expression by 2 μM but not by 40 nM GEN suggests effects on the differentiated progenitor cells, given that CSC lacked ER and PR expression (45,46). Thus, the apparent discrepancies in the dose-dependent effects of GEN on mammosphere formation and gene expression may be a function of the distinct responses to GEN of SC and the niche cells that comprise the tumor bulk.

The present study also demonstrated that bioactive components in systemic circulation resulting from BB consumption can inhibit self-renewal of the CSC-like subpopulation. Although BB phytochemicals have been previously demonstrated to reduce cell proliferation, enhance apoptosis and prevent epithelial–mesenchymal transition of breast cancer cell lines (47), the findings reported here are novel for several reasons. First, the mammosphere formation by sera from BB-fed mice, whereas an ex vivo measurement, provides support for future feeding trial in rodent models to assess in vivo efficacy of consuming BB and other fruits with similar polyphenolic profiles to limit CSC self-renewal. Second, polyphenolic acid metabolites in BB such as hippuric acid may serve as candidate agents for targeting SC, suggesting their value as supplements for women being treated for the disease. Finally, higher BB consumption leading to supra-physiological levels of several polyphenolic acids may not be necessarily beneficial, as shown by the loss of functional response with the highest dose tested. Interestingly, the selected mixture of polyphenolic acids evaluated here preferentially inhibited mammosphere formation of the highly metastatic breast cancer cell line MDA-MB-231 and of the CD44+/CD24⁻/ESA⁺ subpopulation of MDA-MB-231 cells and was ineffective in MCF-7 cells and primary epithelial cells isolated from Wnt-Tg tumors. This was convincingly demonstrated for MCF-7 cells cotreated with BB+GEN, wherein sphere formation inhibitory activity of the treatments could be solely attributed to 40 nM GEN. Since BB sera was effective in inhibiting mammosphere formation in both cell lines, data collectively suggest that other polyphenolic acids or bioactive components targeting the luminal progenitor subpopulation were missing in the BB phenolic acid mixture used in this study. BB phytochemicals have been shown to exert their anti-growth and antimetastatic actions through modulation of the PI3K/Akt/mTOR pathway in MDA-MB-231 cells (47), however, inhibition of mammosphere formation reported here for hippuric acid did not appear to involve PTEN. Taken together, these findings imply that hippuric acid may exert its actions through mechanisms distinct from those of...
the isoflavone GEN and invoke the participation of other SC/progenitor signaling pathways, the latter consistent with the recent demonstration that total loss of PTEN was insufficient to promote anchorage-independent growth of mammary epithelial cells (48).

In conclusion, this study demonstrates that bioactive components in foods conferring dietary benefits to the human population and which are highly bioavailable after regular consumption, can limit the expansion of SC-like and progenitor cells that promote tumor development, progression and recurrence. Whereas earlier studies have reported on the anti-inhibitory effects of dietary factors on breast cancer SC expansion (49,50), our results are the first to demonstrate that dietary factors may display selectivity in inhibiting mammosphere formation and by extension, SC renewal from distinct breast cancer subtypes. Our studies will facilitate the identification of dietary factors for the design of novel targeted therapies for potential translation in the clinics.

Supplementary material

Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/.

Funding

This work was supported by the United States Department of Agriculture grant [CRIS 6251-5100002-06S, Arkansas Children’s Nutrition Center to R.C.M.S.]; Arkansas Children’s Hospital Children’s University Medical Group (award to R.C.M.S.); Department of Defense Breast Cancer Research Program Pre-doctoral Fellowship (W81XWH-08-1-0548 to R.C.M.S.) and Department of Defense Breast Cancer Research Program Pre-doctoral fellowship (W81XWH-10-1-0047 to O.M.R.).

Acknowledgements

We thank Dr F.A. Simmen, Dr J.-R. Chen and laboratory members for helpful discussions and critical reading of this manuscript.

Conflict of Interest Statement: None declared.

References

42. Blick,T. et al. (2010) Epithelial mesenchymal transition traits in human breast cancer cell lines parallel the CD44(hi)/CD24 (lo−) stem cell

Received July 6, 2011; revised December 12, 2011; accepted December 27, 2011