Low Concentrations of the Soy Phytoestrogen Genistein Induce Proteinase Inhibitor 9 and Block Killing of Breast Cancer Cells by Immune Cells

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The risks and benefits of diets and supplements containing the estrogenic soy isoflavone genistein are not well established. We report that 10 nM genistein potently induces the granzyme B inhibitor, proteinase inhibitor 9 (PI-9) in MCF-7 human breast cancer cells. By inducing PI-9, genistein inhibits the ability of human natural killer (NK) cells to lyse the target breast cancer cells. In ERαHA cells, stably transfected MCF-7 cells, which contain elevated levels of estrogen receptor-α (ERα), 100 pM genistein or 17β-estradiol potently induce PI-9 and prevent NK cells from killing the target breast cancer cells. The concentrations of genistein that fully induce PI-9 in MCF-7 cells, and in ERαHA cells, are far lower than those previously reported to elicit estrogenic responses through ERα. Because 4-hydroxytamoxifen, raloxifene, and ICI 182,780/Faslodex all block genistein induction of PI-9 and elevated levels of ERα enhance induction of PI-9, genistein acts via ERα to induce PI-9. Increasing levels of ERα in breast cancer cells results in a progressive increase in induction of PI-9 by genistein and in the cell's ability to evade killing by NK cells. Moderate levels of dietary genistein and soy flour effectively induce PI-9 in human breast cancers grown in ovariectomized athymic mice. A significant population consumes levels of genistein in soy products that may be high enough to induce PI-9, perhaps potentiating the survival of some preexisting breast cancers by enabling them to evade immunosurveillance. (Endocrinology 149: 5366–5373, 2008)

ESTROGENS CONTRIBUTE to the development of breast cancers in part by stimulating the proliferation and metastases of tumor cells (1, 2). We and others recently described a different action of estrogens likely to contribute to tumor development, blocking the ability of immune cells to induce apoptosis of target cancer cells (3–6). The immune cells, cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, recognize and destroy early stage developing tumors in a process sometimes referred to as immunosurveillance (7). Human CTLs and NK cells lyse target cancer cells and virally infected cells primarily by elaborating particles called granules containing the protease granzyme B, other granzymes, and perforin (8, 9). In estrogen receptor-α (ERα)-containing breast and liver cancer cells, estrogens strongly induce the granzyme inhibitor, proteinase inhibitor 9 (SerpinB9, PI-9). By inducing PI-9 in cancer cells, estrogens interfere with the ability of immune system cells to induce apoptosis of human cancer cells (3, 4). We proposed a graded response in which expression of increasing levels of PI-9 in a target cancer cell progressively blocks NK- and CTL-induced apoptosis through the granzyme system (3) and also blocks apoptosis through the secondary Fas/Fas ligand apoptosis pathway (5, 10).

We considered potential sources of estrogens that might induce PI-9 and enable breast cancer cells to evade immunosurveillance. Genistein is an estrogenic soy isoflavone that structurally mimics endogenous 17β-estradiol (E2) (11). In the United States, consumption of soy-based products has increased, perhaps because it is thought they may contribute to the lower rate of incidence of breast cancer seen in Asian countries where soy consumption is high. Epidemiological studies suggest that preadolescent consumption of soy protein correlates with a decreased risk of breast cancer (12). The effect of estrogenic soy isoflavones on the growth and progression of breast cancer has been controversial (13). Low concentrations of genistein stimulate the growth of ERα-containing breast cancer cells in vitro and stimulate growth of preexisting estrogen-dependent breast cancer in mouse xenografts (14–17). High concentrations (>10 μM) genistein are thought to inhibit growth of cancer cells by inhibiting tyrosine kinases (18–21).

In most assays, the reported estrogenicity of genistein in cells containing ERα is quite low (1,000–100,000-fold lower than E2) (22, 23). A recent microarray study in MCF-7 cells found that 3 μM genistein and 30 pm E2 produce similar overall levels of expression of estrogen-regulated mRNAs (23). Another recent study found that 6 nm genistein did not induce ERα-regulated genes in MCF-7 cells (24).

In contrast, we find that 10 nm genistein maximally induces PI-9 mRNA and protein in MCF-7 cells, thereby inhibiting NK cell-induced apoptosis. In ERαHA cells, which express elevated levels of ERα (3, 25), E2 and genistein show similar potency, and 100 pm genistein fully induces PI-9 and elicits a nearly complete block of NK cell-induced apoptosis.
We also found that dietary genistein and soy flour induce PI-9 in solid tumors in the MCF-7 mouse xenograft model.

Materials and Methods

Cell lines and cell culture

MCF-7 human breast cancer cells were cultured in MEM containing 5% calf serum and in phenol red-free MEM containing 5% charcoal dextran-treated calf serum for at least 4 d before experiments. The tet-inducible MCF-7 cells, ERαHa cells, were grown as described (3). NK92 cells and NKL cells were maintained as we recently described (3).

Quantitative RT-PCR

PI-9 mRNA levels were analyzed by quantitative RT-PCR. Primers were designed using Primer Express software (Applied Biosystems, Foster City, CA). PI-9 primers were forward 5'-TGGAAATGACCGTGTTTGACGAA-3' and reverse 5'-CATCTGACTGGCCTTGTGCT-3'. Actin primers were forward 5'-AGGCACCCACCTCCTCTCAA-3' and reverse 5'-AATGCT1ACATCTCCCCGTTG-3'. pS2 primers were forward 5'-ACCAGCACCACAGACGAC-3' and reverse 5'-CTGGTTGTGACGCAGGAC-3'. RNA extraction and cDNA synthesis were as we recently described (3). Briefly, quantitative RT-PCR was carried out in 96-well plates using SYBR Green PCR kit (Applied Biosystems), and samples were amplified with a Bio-Rad iCycler system (Bio-Rad, Hercules, CA). The fold change in expression of each gene was calculated using the ∆∆Ct method with actin mRNA as the internal control.

Western blotting

Whole-cell extracts were prepared in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10−6 m sodium orthovanadate, 10 μg/ml phenylmethylsulfonyl fluoride, and 30 μl/ml aprotinin). For Western blotting, cell lysates containing 5 μg protein were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Because actin is almost the same size as PI-9, calnexin was used as the loading control. Blots were incubated with the appropriate secondary antibodies, and signals were detected by PhosphorImager analysis using ECL Plus (Amersham, Piscataway, NJ).

Cell-mediated cytotoxicity assay

The time-resolved fluorescence assay produces results similar to the well-established chromium release assay (3, 26). The assay was carried out following the supplier's protocol with a few modifications. Target cells were incubated at 106 cells/ml in phenol red-free MEM plus 5% charcoal dextran-treated calf serum with BATDA (PerkinElmer, Welle
cy, CA). PI-99 cells were maintained as we recently described (3). NK92 cells and NKL cells were maintained as we recently described (3).

MCF-7 xenograft studies

MCF-7 cells (27), used in xenografts, represent a widely used animal model for studies of the role of estrogen and genistein in breast cancer (14, 16, 28). Female athymic BALB/c (nude) mice were purchased from Charles River Laboratories (Wilmington, MA) and acclimated for a week. Mice were ovariectomized at 21 d of age by the vendor and acclimated for a week. Mice were ovariectomized at 21 d of age by the vendor and allowed to recover for 7 d. All procedures were conducted with approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois at Urbana-Champaign.

To prepare estrogen pellets, 2 mg E2 and 18 mg cholesterol were mixed and packed into a pellet about 4.5 mm in diameter and 2.5 mm in depth. The E2 pellet was implanted sc in the interscapular region of each mouse 1 wk before MCF-7 cells were injected into the animal.

American Institute of Nutrition 93 growth diet (AIN-93G) semipurified phytoestrogen-free diet (Dyets, Bethlehem, PA) was selected as a base diet. Soy oil was removed from all diets and corn oil added to eliminate any additional components of soy being added to the diets. Treatment animals were fed AIN-93G diet plus genistein at 500 mg/kg (500 ppm) or a diet containing 20% soy protein, which contains approximatively 400 ppm genistein (29).

Analysis of tumor growth

One week after the estrogen pellets were inserted into mice, MCF-7 cells were harvested using 500 μl trypsin-EDTA (0.5% Trypsin, 5.3 mmol/liter EDTA-4Na) (Life Technologies, Inc.-BRL, Grand Island, NY) per 100-mm culture plate. Cells were adjusted to 1×106 cells per 40 μl Matrigel (Collaborative Biomedical Products, Bedford, MA) and injected at 40 μl per site into each of the two flanks of the athymic mice. Tumors were grown until their average cross-sectional area reached 38 mm2. One week before grouping, animal diets were switched to the casein-based AIN-93G diet. Mice were divided into three treatment groups (13–14 mice per group): negative control (NC), positive control (PC, estradiol control), and 500 ppm genistein treatment groups. Estradiol pellets were removed from all mice except the PC group. Mice in the genistein group started with 500 ppm genistein diet treatment. Tumor growth and body weight were measured weekly for 23 wk. The cross-sectional area of tumor was determined by using the formula [length/2 × width/2] (30). Food intake was measured throughout the study. At the end of the study, mice were anesthetized and killed by cervical dislocation. Tissues and blood samples were collected for analysis. Tumors were removed and stored in liquid nitrogen for RNA analysis.

Statistical analysis

The results are reported as mean ± SEM. We compared the ability of NK92 and NKL cells to induce apoptosis of control and genistein-treated ERαHa cells expressing different levels of ERα. For each panel of Fig. 3, we used the single-tailed t test to compare apoptosis in control cells and cells treated with three ratios of NK cells (P < 0.05 for control vs. genistein-treated cells in Fig. 3, A, C, and D-F; panel B, P > 0.05).

Results

Nanomolar concentrations of genistein induce PI-9 in MCF-7 breast cancer cells

Using quantitative RT-PCR to determine PI-9 mRNA levels, we examined the concentrations of the soy isoflavones genistein and equol (−) (the active metabolite of diadzein) (31) and the plant estrogen coumestrol required to induce PI-9 mRNA in MCF-7 human breast cancer cells. Induction of PI-9 by E2 was used for comparison. The concentrations of equol (−) and coumestrol required to maximally induce PI-9 mRNA (100 nm) were 1000 times higher than the 100 pm E2 required for maximal induction. However, only 10 nm genistein, a level likely to be observed in the blood of women consuming soy or soy-based products (32), was required to maximally induce PI-9 mRNA. In contrast, a 1000-fold higher concentration of genistein, 10 μm, was required to induce pS2 mRNA (Fig. 1A, dashed line). The high concentration of genistein required to induce pS2 mRNA is consistent with earlier work (33). Previously, 200 pm E2 was shown to potently induce pS2 mRNA (33).

Because of genistein's high potency as an inducer of PI-9, we explored whether binding of genistein in the ligand binding pocket of ER is required. The selective ER modulators (SERMs) 4-hydroxysteramoxifen (OHT), the active metabolite of

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to elevate the ERα level 7- to 8-fold (Fig. 2, A and B). When the ERα level is about 3-fold higher than in wild-type MCF-7 cells, genistein’s potency equals that of E2, and both maximally induce PI-9 mRNA at 100 pm. The concentrations of equol (–) and coumestrol required to maximally induce PI-9 mRNA are 100- to 1000-fold higher (Fig. 2C). Increasing ERα to 7- to 8-fold above the level in MCF-7 cells increased the induction of PI-9 mRNA to approximately 250-fold without significantly altering the concentration of genistein required for induction (Fig. 2D). Western blot analysis demonstrated that 100 pm genistein elicits a robust induction of PI-9 protein in the ERαHA cells expressing the highest levels of ERα (Fig. 2E).

**Increased induction of PI-9 progressively blocks NK cell-mediated apoptosis**

We next tested whether induction of PI-9 by genistein reduces the ability of human immune cells to induce apoptosis of breast cancer cells. Human NK cells and CTLs lyse their target cells by similar mechanisms, so we used established NK cell lines as killer cells. NK cells target MCF-7 cells through the granzyme pathway, not the Fas/Fas ligand pathway (3). To test the effect of genistein induction of PI-9 on NK cells inducing cell death via diverse death pathways, we used NK92 cells, which contain primarily granzyme B, and NKL cells, which reportedly contain mostly granzyme A (36, 37). Granzyme A induces rapid cell death by a pathway distinct from the granzyme B pathway (38), and its sensitivity to *in vitro* inhibition by PI-9 has not been established. We compared the ability of NK92 and NKL cells to induce apoptosis of control and genistein-treated MCF-7 and ERαHA cells expressing different levels of ERα. For each panel of Fig. 3, we used the one-tailed Student’s *t* test to compare apoptosis in control cells and genistein-treated cells with three ratios of NK cells (*P* < 0.05 for control vs. genistein-treated cells in A, C, and D–F; *P* > 0.05 in B).

Genistein treatment induced levels of PI-9 in MCF-7 cells sufficient to block NK92 cell-induced apoptosis (Fig. 3A) but not NKL cell-induced cell death (Fig. 3B). We recently reported a similar finding in MCF-7 cells treated with E2 (3). In ERαHA cells expressing 2- to 3-fold higher levels of ERα than MCF-7 cells (Fig. 2B), genistein induces a higher level of PI-9 (Fig. 2E). This increased level of PI-9 inhibited both NK92 (Fig. 3C) and NKL-induced cell death (Fig. 3D). The very high level of PI-9 induced by genistein in ERαHA cells expressing 7- to 8-fold more ERα than MCF-7 cells (Fig. 2E) resulted in a nearly complete inhibition of both NK92 cell-induced cell death (Fig. 3E) and NKL-mediated cytotoxicity (Fig. 3F). These data demonstrate that increasing levels of ERα in breast cancer cells elicit enhanced genistein induction of PI-9. The enhanced genistein induction of PI-9 results in progressive inhibition of NK cell-induced cell death.

**Dietary genistein and soy products induce PI-9 mRNA in MCF-7 tumors in ovariectomized athymic mice**

The studies in Figs. 1–3 used human breast cancer cells in culture to demonstrate that 100 pm and 10 nm genistein, concentrations of genistein previously thought to be too low to elicit significant biological effects, potently induce PI-9 and...
block immunosurveillance. We wished to test whether genistein consumed from more typical dietary sources could induce PI-9 in tumors. Although studies testing genistein effects on endogenous breast tumors in mice might seem attractive, they are not appropriate. The mouse serpin whose amino acid sequence and tissue distribution most closely resembles human PI-9 is serine protease inhibitor 6 (SPI-6). The protein coding regions of PI-9 and SPI-6 are about 60% identical. However, the promoter regions of human PI-9 and mouse SPI-6 show virtually no sequence homology, and the mouse and rat SPI-6 promoter regions do not contain the downstream estrogen-responsive unit (ERU) critical for estrogen induction of PI-9 in human cells (39–41). In contrast, the ERU is highly conserved in the human, chimpanzee, and Rhesus monkey PI-9 genes. In studies using control and E2-treated rat liver (supplied by Prof. J. Katzenellenbogen), E2 did not influence SPI-6 levels (Krieg, S., J. A. Katzenellenbogen, and D. J. Shapiro, unpublished). There are also important differences between mouse and human granzyme B and their granzyme inhibitors (42, 43). We therefore tested whether mice fed genistein could induce the same levels of PI-9 in MCF-7 xenografts that we showed inhibit NK cell-mediated lysis of MCF-7 cells in our in vitro studies.

To mimic the slow tumor growth observed in postmenopausal women with breast cancer, we used a level of dietary genistein previously shown to induce a moderate rate of tumor growth (17). MCF-7 cell tumors were established by injection of MCF-7 cells and brief exposure of the mice to estrogen in estrogen pellets. In one group of mice, continued exposure to high concentrations of E2 in the estrogen pellets resulted in rapid growth of the MCF-7 cell tumors (Fig. 4A, positive control (PC estradiol)). The other groups of mice received 500 ppm genistein in their diet, a level of genistein that results in much slower tumor outgrowth over about 5 months (Fig. 4A, Gen 500), or diets supplemented with soy flour (29). It required about four times longer for the tumors in mice fed genistein to reach a 100-mm² surface area than for the tumors in mice exposed to high E2 (23 vs. 4–5 wk, Fig. 4A).

Because our affinity-purified anti-PI-9 detects both human PI-9 and its closest mouse relative, SPI-6, and RT-PCR indicated the presence of significant SPI-6 mRNA in the tumor samples (data not shown), we used quantitative RT-PCR to specifically determine levels of PI-9 mRNA in the MCF-7 tumor samples. In MCF-7 cells, PI-9 mRNA is rapidly induced by estrogens to high levels, peaks at about 3–4 h, and then declines to an induction of severalfold that is maintained for at least a few days (3). We used cultured MCF-7
cells as control cells because MCF-7 tumors require estrogen for growth, and the regressing tumor samples did not contain significant numbers of viable MCF-7 cells. Because the tumors were continuously exposed to E2, genistein, or genistein in soy products, we compared levels of PI-9 mRNA in the frozen tumor samples to PI-9 mRNA levels in control MCF-7 cells and in MCF-7 cells treated with E2 or genistein for 24 h, followed by incubation with NK92 effector (E) cells (A) or NKL effector cells (B) at different effector cell/target cell ratios. Cytotoxicity assays were performed as described in Materials and Methods and Ref. 3. C and D, Genistein protects ERαHA cells expressing high levels of ERα against NK92 cell and NKL cell-mediated killing. ERαHA cells were treated with ethanol vehicle or with 0.5 μg/ml DOX plus 100 μM genistein for 48 h, followed by the cytotoxicity assay. E and F, In ERαHA cells expressing very high levels of ERα, genistein nearly abolishes NK92 and NKL cell-induced cell death. ERαHA cells were treated with ethanol vehicle or with 1.0 μg/ml DOX plus 100 μM genistein for 48 h, followed by the cytotoxicity assay. Data in A–F represent the mean ± SEM for three separate experiments. For each panel, we used the single-tailed t test to compare apoptosis in control cells and cells treated with three ratios of NK cells (P < 0.05 for control vs. genistein-treated cells in A, C, and D–F; B, P > 0.05).

Discussion

In contrast to the pS2 gene and most other genes that require micromolar genistein for induction (23, 24, 33), induction of PI-9 requires nanomolar to picomolar concentrations of genistein. Several lines of evidence indicate that the induction of PI-9 in MCF-7 cells, and in ERαHA cells, is mediated by genistein acting via ERα. Although genistein binds with much higher affinity to ERβ than to ERα and lower concentrations of genistein activate transcription through ERβ (24, 44), MCF-7 cells contain negligible ERβ (24). Also, in ERαHA cells, which are MCF-7 cells stably transfected to express additional ERα under the control of a tetracycline-regulated promoter, the concentrations of genistein required for robust induction of PI-9 is reduced from 10 nM in MCF-7 cells to 100 pM. Several studies indicate that G protein-coupled receptor 30 (GPR30) is a potential membrane ER (34). However, GPR30 is an integral membrane protein thought to participate mostly in nongenomic effects of estrogens. Because OHT is an agonist on GPR30 and ICI 182,780 also displays some agonist effects (34), our observation that a 100-fold molar excess of OHT, raloxifene, and ICI 182,780 all block genistein induction of PI-9 (Fig. 1B) is inconsistent with induction of PI-9 by genistein acting through GPR30. Although genistein is a tyrosine kinase inhibitor, this requires micromolar concentrations of genistein, not the nanomolar to picomolar concentrations we use. Increasing levels of ERα in ERαHA cells enhance the magnitude of genistein induction of PI-9 and
reduce the concentrations of genistein required for maximal induction (Fig. 2). Thus, our data support the view that genistein induces PI-9 by binding in the ligand binding pocket of ERα.

The PI-9 gene’s sensitivity to activation by low concentrations of genistein may result from the ability of genistein to induce an ER conformation highly conducive to binding to the PI-9 ERU. We previously reported that binding of intracellular ER to the ERU that controls PI-9 transcription involves a balance between ER conformations induced by the DNA sequence and the bound ligand (41). Because the PI-9 ERU contains two directly adjacent weak ERα binding sites (39, 41), it is also possible that the two ERα dimers mutually stabilize each other’s binding to the ERU, making the PI-9 gene highly responsive to small increases in the intracellular concentration of ER. Although the factors that make the PI-9 gene sensitive to activation by extremely low concentrations of genistein are unclear, the concentrations of genistein that fully induce PI-9 in MCF-7 cells, and in ERαHA cells, are far lower than those previously reported to elicit estrogenic effects through ERα (23, 24, 33).

RNA interference knockdown of PI-9 completely restored the ability of cytolytic lymphocytes to induces cell death in estrogen-treated MCF-7 cells and hepatoblastoma cells (3, 4). Our observation that lower concentrations of genistein (100 ppm) in MCF-7-derived ERαHA cells, which express additional ERα, more effectively induce PI-9 and inhibit NK cell apoptosis than the higher genistein concentration (10 nm) in MCF-7 cells makes it extremely unlikely that genistein’s ability to inhibit NK cell-induced apoptosis through the granzyme system is mediated by genistein’s ability to inhibit tyrosine kinases or by an as yet undiscovered activity of genistein. Thus, genistein’s ability to inhibit NK cell-mediated apoptosis through the granzyme pathway likely results from its acting through ERα to induce the granzyme inhibitor PI-9.

Numerous studies show an association between the risk of breast cancer and elevated blood levels of estrogen. Studies showing that estrogen-replacement therapy increases the relative risk of breast cancer have led to increased interest and use of soy products as an alternative therapy to treat peri- or postmenopausal symptoms. Prolonged dietary supplementation with soy makes about 10–20 nm genistein available to cells (32). Because 10 nm genistein fully induces PI-9 and inhibits NK92-induced apoptosis of MCF-7 breast cancer cells, circulating concentrations of genistein that can be reached during isoflavone supplementation, or by consuming a diet rich in soy isoflavones, are probably sufficient to robustly induce PI-9 and thereby reduce the ability of cytolytic lymphocytes to kill target breast cancer cells. Because 10 nm genistein fully induces PI-9 and inhibits NK92-induced apoptosis of MCF-7 breast cancer cells, circulating concentrations of genistein that can be reached during isoflavone supplementation, or by consuming a diet rich in soy isoflavones, are probably sufficient to robustly induce PI-9 and thereby reduce the ability of cytolytic lymphocytes to kill target breast cancer cells. Because 100 pm E2 maximally induces PI-9 (Fig. 2), and 50 pm E2, which is the average circulating level of estrogen in postmenopausal women (45), partially induces PI-9, genistein consumed from nearly ubiquitous dietary soy may enhance the induction of PI-9 in breast cancer cells in a large cohort of postmenopausal women. Two populations, infants and young children and breast cancer patients undergoing treatment with aromatase inhibitors, have very low circulating estrogen levels that should not induce PI-9 (46–48). In these populations, dietary soy may contribute sufficient genistein to induce PI-9 in some cells and enable the cells to evade immunosurveillance. The consequences of genistein altering the susceptibility of target cells to lysis by cytolytic lymphocytes are probably influenced by both the duration and time of genistein exposure. In some cases, especially in liver cells, some of the pathology that leads to tumor formation is based on inflammatory processes that attract cytolytic lymphocytes, which then damage the target cells (49). By inducing PI-9, genistein might protect cells against cytolytic lymphocyte-mediated damage initiated by inflammatory processes. However, once a cell is transformed, exposure to genistein...
will induce PI-9 and reduce cytolytic lymphocyte-mediated immnosurveillance of the newly transformed cells.

A small subset of breast cancers express much higher levels of ERα than are seen in MCF-7 breast cancer cells. This generally results in a poor prognosis (25, 50). ERαHA cells serve as a potential model for these types of cancers (25). In these cells, 100 pm genistein potently induced PI-9, resulting in a nearly complete inhibition of NK cell-mediated lysis of the ERαHA cells. This is the lowest concentration of genistein shown to elicit a biological effect and suggests that for this small subgroup of breast cancer patients, it may be appropriate to consider issues related to levels of soy consumption. Altered expression of PI-9 is associated with a poor prognosis in several tumors (51–53). Because genistein from dietary soy reportedly enhances the growth of preexisting breast cancers (15, 16), the possibility that genistein induction of PI-9 plays a role in tumor progression in breast cancer patients whose tumors contain very high levels of ERα warrants further attention.

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