Molecular effects of genistein on estrogen receptor mediated pathways

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Genistein, a component of soy products, may play a role in the prevention of breast and prostate cancer. However, little is known about the molecular mechanisms involved. In the present study, we examined the effects of genistein on the estrogen receptor positive human breast cancer cell line MCF-7. We observed that genistein stimulated estrogen-responsive pS2 mRNA expression at concentrations as low as $10^{-8}$ M and these effects can be inhibited by tamoxifen. We also showed that genistein competed with $[^3H]$estradiol binding to the estrogen receptor with 50% inhibition at $5 \times 10^{-7}$ M. Thus, the estrogenic effect of genistein would appear to be a result of an interaction with the estrogen receptor. The effect of genistein on growth of MCF-7 cells was also examined. Genistein produced a concentration-dependent effect on the growth of MCF-7 cells. At lower concentrations ($10^{-8}$ to $10^{-6}$ M) genistein stimulated growth, but at higher concentrations ($>10^{-5}$ M) genistein inhibited growth. The effects of genistein on growth at lower concentrations appeared to be via the estrogen receptor pathway, while the effects at higher concentrations were independent of the estrogen receptor. We also found that genistein, though estrogenic, can interfere with the effects of estradiol. In addition, prolonged exposure to genistein resulted in a decrease in estrogen receptor mRNA level as well as a decreased response to stimulation by estradiol.

Introduction

In epidemiological studies, an increased intake of soy products is associated with a decreased risk of hormone-dependent cancers (1,2). In rodents, ingestion of soy products was found to inhibit mammary tumorigenesis (3). It has been suggested that genistein, an isoflavone abundant in soy products, may contribute to the chemopreventive effects of soy products for hormone-dependent cancers (2,4). However, the molecular mechanisms by which genistein exerts its effects remain poorly understood. This is not due to a lack of proposed and identified effects. On the one hand, genistein has been shown to stimulate proliferation, presumably through estrogen-like effects (5). On the other hand, it is considered to be antiproliferative, presumably due to inhibition of tyrosine kinases (6–8) and topoisomerase (9). Until its mechanisms of action are better understood, the chemopreventive potential of genistein is unlikely to be fully realized.

In the present study, we focused on the effect of genistein on the estrogen receptor (ER) pathway. Using the human breast cancer MCF-7 cells, we examined the effects of RNA on: (i) the expression of pS2 mRNA; (ii) the displacement of estrogen from binding to the ER; (iii) cellular proliferation; and (iv) regulation of the ER. The results from these studies were used to address the following questions. (i) Is genistein estrogenic? (ii) Could genistein be anti-estrogenic? (iii) What contributes to the paradoxical observation that genistein stimulates both proliferative and anti-proliferative responses?

Materials and methods

Chemicals

Genistein was purchased from Sigma Chemical Co. (St Louis, MO). Quercetin was purchased from Indofine Chemical Co. (Somerville, NJ). Tamoxifen was purchased from Aldrich Chemical Co. (Milwaukee, WI). These compounds were >95% pure. All other chemicals were from the best sources available.

Cells and cell culture

MCF-7 cells were maintained in Medium A [RPMI 1640 with 2 mM L-glutamine, hydrocortisone (3.5 ng/ml), insulin (1.5 ng/ml), penicillin (100 U/ml) and streptomycin (100 mg/ml)] with 5% fetal bovine serum in 75 cm² flasks (Falcon) and grown in the presence of 5% CO₂ in air at 37°C. One week before initiation of the experiment, cells were switched to Medium A supplemented with 5% charcoal dextran-treated fetal bovine serum (CDS). Cells were grown to confluence and passed using trypsin–EDTA. Viability cells were counted by Trypan blue exclusion. A day prior to treatment the cells were switched to Medium B [phenol-red free RPM1 1640 containing 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 mg/ml) and 5% CDS].

Probe for pS2 and ER

Digoxygenin (DIG)-labeled pS2 cRNA probe (10) was synthesized using a nucleic acid labeling kit following the manufacturer’s instructions (Boehringer Mannheim Biochemicals, Indianapolis, IN). For production of the DIG-ER cRNA probe, plasmid pOR3 carrying the ER cDNA (11) was obtained from the American Type Culture Collection. The pOR3 was digested with EcoRI and a 1.35 kb cDNA fragment coding for the ER was inserted into the EcoRI site of plasmid pGEM-4Z. The resulting plasmid was designated pGEM/ER. DIG-labeled ER cRNA probe was generated from NarI linearized pGEM/ER using the SP6 RNA polymerase following the procedure described previously (10).

Total RNA isolation, Northern blot hybridization and detection

For isolation of total RNA, MCF-7 cells were grown in six-well Costar plates (1 X 10⁶ cells/well in 3 ml of Medium B) and isolated as described previously (10). RNA was resuspended in 70–100 µl DEPC-treated water, and 10 µl of RNA was routinely used for Northern blot analysis. For detection of mRNA, 10 µl of total RNA was separated on a 1% agarose formaldehyde gel and transferred to nylon membrane (Boehringer Mannheim) using the PhastBlot Pressure Blotter (Stratagene, La Jolla, CA). The RNA was fixed onto the membrane by UV crosslinking in a Stratalinker (Stratagene) for 3 min at the highest power. The membrane was then hybridized with DIG-labeled RNA probe and detected by a chemiluminescence nucleic acid detection method (NEN, Boston, MA). The membrane was exposed to X-ray film (Kodak, Rochester, NY), the intensity of the pS2, ER, G3PDH or β-actin mRNA bands were quantitated by densitometry (Anatech Uniscan, Newark, DE). Densitometer readings were normalized for either G3PDH or β-actin RNA content and expressed as relative expression level.

Growth assay

Cell growth was determined by the sulforhodamine colorimetric assay as described by Skelan et al. (12) except that the cells were seeded in 24-well
plates instead of 96-microwell plates. Routinely, 1 × 10⁶ cells in 1 ml of Medium B were seeded to each well of a 24-well plate (Costar, Cambridge, MA). Twenty-four hours after plating, either genistein or vehicle was added. The medium was changed every 24 h and the test compounds were replenished with each medium change. After color development, aliquots were pipetted into a 96-well microtiter plate and the intensity of the developed color was measured at 570 nm using an ELISA microplate reader (Molecular Devices, Menlo Park, CA).

**ER binding study**
MCF-7 cells were grown to confluence in 150 cm² flasks (Becton Dickinson Labware, Lincoln Park, NJ) and cultured in Medium A supplemented with 5% fetal bovine serum at 37°C in the presence of 5% CO₂ in air. The cells were rinsed twice with PBS, scraped off in 5 ml of PBS and pelleted at 1500 r.p.m. for 10 min. After aspiration of the supernatant, the cell pellets were suspended in lysis buffer consisting of 10 mM Tris–HCl, pH 7.4, 1.5 mM EDTA and 0.5 mM diithiothreitol. The cell suspension was homogenized using a Tekmar Tissumizer (Tekmar Co., Cincinnati, OH) for 45 s. The homogenate was then centrifuged at 40 000 r.p.m. for 50 min and the supernatant saved. After determination of the protein concentration, the supernatant was used for ER binding assays. ER binding assay was performed essentially as described by McGuire (13). Briefly, the ER binding studies were carried out as follows: 200 μl of the cytosol fraction (2.0 mg/ml protein) was incubated with 10⁻⁹ M [³H]estradiol (New England Nuclear, Boston, MA) for 18 h at 4°C. Following incubation, 0.5 ml of dextran-coated charcoal was added to the assay mixture, and incubated at 4°C for 30 min with vortexing at 5 min intervals. The assay mixture was then centrifuged at 2000 g for 10 min and 0.5 ml of the supernatant was removed for measurement of the bound [³H]estradiol by liquid scintillation spectrometry. For competition experiments, binding assays were run as described above in the presence of increasing amounts of competitor (0–10⁻⁶ M) while the concentration of estradiol was held constant at 10⁻⁹ M.

**Results**

**Effect of genistein on pS2 mRNA expression and estradiol binding to ER**
The structure of the compounds used in the study is illustrated in Figure 1. To characterize the estrogen-like property of genistein we monitored the expression of estrogen-dependent pS2 mRNA using a chemiluminescence detection method reported previously (10). As illustrated in Figure 2, stimulation of pS2 mRNA expression occurred in a concentration-dependent fashion. The stimulatory effect of genistein on pS2 mRNA expression was observed at concentrations as low as 10⁻⁸ M. As illustrated in Figure 3(A), the stimulation of pS2 expression by genistein (10⁻⁷ M) could be blocked by the addition of tamoxifen (10⁻⁵ M). In addition, actinomycin D at a concentration of 3 μg/ml inhibited the genistein-stimulated pS2 expression (Figure 3B). These results support the hypothesis that genistein acts through an ER-mediated transcriptional event.

To characterize further the interaction between genistein and the ER pathway, we examined the ability of genistein to compete with [³H]estradiol (10⁻⁹ M) for binding to the ER. As shown in Figure 4, genistein can compete with estradiol for binding to the ER. In contrast, quercetin, which does not stimulate pS2 expression (10), did not compete with estradiol for binding to ER. The concentration required for genistein to produce a 50% inhibition of binding was 5 × 10⁻⁷ M. These results, together with the results from the pS2 expression assay, supported the interpretation that genistein induces an estrogenic response by interacting with the ER.

**Anti-estrogenic effects of genistein**
It is known that anti-estrogens such as tamoxifen can also act as weak estrogens (14). Hence, like tamoxifen, genistein may

Figs. 2. Concentration-dependent stimulation of pS2 expression by genistein. MCF-7 cells were cultured in Media B in the presence of various concentrations of genistein (10⁻⁹–10⁻⁵ M). Total RNA was isolated after 48 h. Northern blot and detection for pS2 and β-actin mRNA was performed as described in Materials and methods. The results are expressed as relative pS2 expression.

Fig. 3. Effects of tamoxifen and actinomycin D on genistein-stimulated pS2 mRNA level. (A) Effects of tamoxifen on genistein-stimulated pS2 mRNA expression. Cells were incubated in the presence of genistein (10⁻⁷ M) and with or without tamoxifen (10⁻⁵ M) for 48 h. Total RNA was isolated after 48 h and pS2 mRNA level determined as described in Materials and methods. (B) Effects of actinomycin D on genistein-stimulated pS2 mRNA expression. Cells were incubated in the presence of genistein (10⁻⁷ M) with or without actinomycin D (3 μg/ml) for 48 h. Total RNA was isolated after 48 h and pS2 mRNA level determined as described in Materials and methods.

Fig. 1. Structure of estradiol, genistein and quercetin.
act both as an agonist and antagonist of the ER-dependent pathway. To address this question, we examined both the short- and long-term interaction of genistein with estrogen. When both genistein and estradiol were added together, the expression of pS2 was lower than when each compound was added alone (Figure 5). These results suggest that genistein may interfere with the effects of estrogen. In addition, under physiological conditions, exposure to genistein may be cyclic and of longer duration (15,16). Daily addition of genistein into culture media may mimic such a situation. Thus, we examined whether pretreatment of MCF-7 cells with genistein affects the response of this cell line to estradiol. Again, using expression of pS2 mRNA as an indicator, we observed that in MCF-7 cells pretreated with genistein (10⁻⁶ M) for 6 days, the stimulatory response to estradiol was lower than control cells (Figure 6). The decreased response to estradiol from long-term exposure of cells to genistein appears to be the result of decreased ER mRNA level. We found that the steady-state ER mRNA level of the cells treated with genistein was 60% of the untreated cells, similar to cells treated with estradiol (Figure 7).

**Effect of genistein on the growth of MCF-7 cells**

In addition to a specific end point mediated through interaction with the ER, the effects of genistein on cellular proliferation was also examined. We monitored the growth of MCF-7 cells in response to genistein at concentrations ranging from 10⁻⁹ to 10⁻⁴ M and found that the effect on proliferation of MCF-7 cells was biphasic. Genistein concentration between 10⁻⁸ and 10⁻⁵ M stimulated the growth of MCF-7 cells (Figure 8). However, at concentrations >10⁻⁵ M, genistein appeared to inhibit proliferation (Figure 8). Since the threshold for the stimulation of proliferation was in the range of concentration for stimulating expression of pS2, we considered that the stimulation of growth was mediated through the ER. Evidence supporting involvement of the ER was found in studies using the ER-negative MDA-MB-231 cells in which genistein did not stimulate growth. However, in these ER-negative cells, the inhibitory effects of genistein (10⁻⁷–10⁻⁴ M) remained (Figure 8).

**Discussion**

The present study focused on genistein’s mechanism of action and on the apparent contradictions in previously published data. The decreased response to estradiol from long-term exposure of cells to genistein appears to be the result of decreased ER mRNA level. We found that the steady-state ER mRNA level of the cells treated with genistein was 60% of the untreated cells, similar to cells treated with estradiol (Figure 7).

**Figure 4. Effects of genistein and quercetin on the binding of [³H]estradiol to the ER.** ER binding assays were performed as described in Materials and methods. Competitors (estradiol, genistein and quercetin) were added at the indicated concentrations with 10⁻⁹ M [³H]estradiol. The results (mean±SE, n = 3) are expressed as a percentage of control.

**Figure 5. Effects of genistein on estradiol-stimulated pS2 mRNA expression.** MCF-7 cells were incubated with genistein (10⁻⁷ or 10⁻⁶ M) alone, estradiol (10⁻¹¹ M) alone, or media containing both genistein and estradiol. Total RNA was isolated after 48 h and pS2 mRNA expression was determined as described in Materials and methods. The results (mean±SE, n = 3) are expressed as relative level of pS2 mRNA expression. Data were analyzed using ANOVA and post hoc comparison using Fisher’s PLSD test. Bars with different letters are significantly different from each other (P < 0.05).

**Figure 6. Effects of long-term exposure to genistein on estradiol-stimulated pS2 mRNA expression.** MCF-7 cells were incubated with genistein (10⁻⁷ or 10⁻⁶ M) alone, estradiol (10⁻¹¹ M) alone, or media containing both genistein and estradiol. Total RNA was isolated after 48 h and pS2 expression was determined as described in Materials and methods. The results (mean±SE, n = 3) are expressed as relative level of pS2 mRNA expression. Data were analyzed using ANOVA and post hoc comparison using Fisher’s PLSD test. Bars with different letters are significantly different from each other (P < 0.05).

**Figure 7. Effect of long-term treatment with genistein on ER mRNA level.** MCF-7 cells were incubated in the presence of 10⁻⁶ M genistein or 10⁻¹⁰ M estradiol for 6 days. Media and test compounds were changed daily. On the 7th day total RNA was isolated and ER mRNA level determined as described in Materials and methods. Data are expressed as relative to control (mean±SE, n = 4). Data were analyzed using ANOVA and post hoc comparison using Fisher’s PLSD test. Bars with different letters are significantly different from each other (P < 0.05).
Fig. 8. Effects of varied concentrations of genistein on growth of ER-positive (MCF-7) and ER-negative (MDA-MB-231) cells. Cells were cultured in the presence of various concentrations of genistein ($10^{-9}$ to $10^{-4}$ M) for 5 days. Cell growth was determined as described in Materials and methods. The results (mean ± SE, n = 4) are expressed relative to cells grown without genistein.

work in this area. In cultured cells, both proliferative and anti-proliferative effects have been ascribed to genistein (5-9). We found that in the ER-positive human breast cancer cell line MCF-7, the concentration-dependent effect of genistein on growth was in fact biphasic. At low ($10^{-8}$ to $10^{-5}$ M) concentrations, genistein stimulated cell growth, while at high ($2.5 	imes 10^{-5}$ to $10^{-4}$ M) concentrations, genistein inhibited cell growth. Since genistein failed to stimulate the proliferation of ER-negative cells, we concluded that the proliferative effect of genistein was mediated through ER. The effects of genistein on pS2 mRNA expression and binding of estradiol to ER provided additional support for this conclusion because: (i) genistein stimulated both proliferation and pS2 expression in a concentration-dependent manner over the concentration range of $10^{-8}$ to $10^{-5}$ M; (ii) the affinity of genistein towards ER ($K_i = 10^{-7}$ M) is in the same concentration range that we observed increased pS2 mRNA expression and cellular proliferation; (iii) stimulation of pS2 mRNA expression by genistein was inhibited by the anti-estrogen tamoxifen.

By contrast, the anti-proliferative effect on MCF-7 cells observed with high concentrations of genistein ($2.5 	imes 10^{-5}$ to $10^{-4}$ M) appeared independent of ER. This growth inhibition was also observed in ER-negative cells in which no growth stimulatory effect was observed at low genistein concentrations. Since the growth inhibitory concentration is in the range for inhibition of tyrosine kinases (6-8) and topoisomerases (9), it is likely that growth inhibition seen in tissue culture at genistein concentrations $>2.5 	imes 10^{-5}$ M may result from perturbation of post-receptor signaling pathways and/or interference with DNA synthesis, and is, in fact, independent of ER. Initially, the aforementioned effects might appear to be involved in the mechanism by which genistein contributes to the decreased risk in hormone-dependent cancers associated with ingestion of soy products. However, this is unlikely since in humans the circulating level of genistein does not exceed $2.5 	imes 10^{-5}$ M (15,16). Even with increased intake of soy products, circulating levels of genistein normally do not exceed $10^{-6}$ M (15,16). It is more likely that the effects of dietary genistein on tumorigenesis are mediated through the ER, for which it has a relatively high affinity ($K_i = 10^{-7}$ M).

Results from the present study provide evidence for the anti-estrogenic effects of genistein. We observed that not only does genistein compete with estradiol for binding to the ER, but it also interferes with the effects of estrogen. When genistein and estradiol were added together in the pS2 expression assay, the expression of pS2 mRNA was lower than when these compounds were added individually. In addition, the duration of exposure to genistein also appeared to be important. MCF-7 cells treated with genistein showed a decrease in the response to estrogen as well as a decrease in ER mRNA levels. This is similar to cells treated with estradiol, as has been described by others (17). Taken together, the results support the idea that genistein at physiologically achievable concentrations may, in fact, interfere with the action of estrogen through direct competition for binding to ER and by reducing ER expression. These effects could be considered anti-estrogenic, consistent with the proposed cancer-preventive effects of soy diet.

In summary, our findings provide support for a model in which phyto-estrogens such as genistein, at physiological concentrations, may exert their effect by modulating estrogenic pathways. This interaction may occur directly through competition with estrogen for binding to ER. In addition, prolonged exposure may result in the reduction of ER expression and hence lead to decreased responsiveness to endogenous estrogens.

References
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