Effects of the Phytoestrogens Genistein and Daidzein on BRCA2 Tumor Suppressor Gene Expression in Breast Cell Lines

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Abstract: A high intake of isoflavones is associated with a reduction of breast cancer risk among Japanese women. The aim of this study was to quantify BRCA2 tumor suppressor gene expression after treatment of cells with the phytoestrogens daidzein and genistein, the main compounds of soy. The effects of 3 µg/ml genistein and 20 µg/ml daidzein on BRCA2 expression were studied in two human mammary tumor cell lines, MCF7 and MDA-MB-231, and one normal human breast epithelial cell line, MCF10a. BRCA2 mRNA was evaluated by quantitative real time RT-PCR and the amount of BRCA2 protein was measured by affinity chromatography. With genistein, we observed a 60% increase of BRCA2 mRNA in MDA-MB-231 and MCF10a, which are, respectively, estrogen receptors α–β+ and α–β–, and no variation in MCF7, which is ERα+/β+. Daidzein had no effect on BRCA2 mRNA expression. The level of BRCA2 protein with both food components also remained unchanged in all three cell lines. This suggests regulation of BRCA2 at the mRNA and protein levels. Treatment with actinomycin D and cycloheximide demonstrated that the increase in BRCA2 mRNA was not blocked by cycloheximide, indicating that de novo protein synthesis was required in MDA-MB-23, although de novo protein synthesis was not required in MCF10a for the genistein. With actinomycin D, genistein had a positive action on the transcriptional level of BRCA2 mRNA in MDA-MB-231 and MCF10a. The use of an anti-estrogen suggested that the action of daidzein and genistein on BRCA2 expression in human breast cell lines might not be mediated through the ER.

Introduction

Two major genes, BRCA1 (1) and BRCA2 (2), have been implicated in inherited predisposition to female breast and ovarian cancers. Germ-line mutations in BRCA1 and BRCA2 are believed to be responsible for 5–10% of all breast cancer cases (3). However, somatic mutations of BRCA1 and BRCA2 are never found in sporadic breast cancers (4,5). Relative to normal breast epithelial cells, BRCA1 mRNA levels in tumors appeared to be down-regulated by a methylation mechanism. In contrast, Collins et al. (6) demonstrated the absence of methylation of the BRCA2 promoter, and Bieche et al. (7) showed significant overexpression of BRCA2 in sporadic breast cancers.

The BRCA2 gene at 13q12-13 is composed of 27 exons and encodes a protein of 3,418 amino acids with no significant homology to any known protein (8,9). The BRCA2 transcript is larger than 10 kb, and its expression has been shown to be cell cycle regulated (10–12). BRCA2 protein appears to reside in the nucleus (13). BRCA2 is expressed ubiquitously, with highest levels in the thymus and testis, and is generally high in rapidly dividing differentiating tissues and most notably during mammary epithelial proliferation and differentiation (14). BRCA2 protein seems to have several roles, including a direct role of DNA repair. Indeed, BRCA2 and RAD51 interact and colocalize in a BRCA1-BRCA2-RAD51 complex, with RAD51 being one of the best-studied proteins involved in eukaryotic double-strand break repair and homologous recombination. An interaction between BRCA2 and RNA polymerase II was shown by Maldonado et al. (15), suggesting a role in transcriptional regulation. Additional evidence for a role in transcriptional regulation was provided by Fuks et al. (16), who showed that BRCA2 interacts with the transcriptional coactivator protein P/CAF (p300/CBP associated factor), which possesses histone acetylase activity. Moreover, BRCA2 itself has histone acetyltransferase activity (17).

The low incidence of breast cancer among Asians may be explained in part by dietary habits. A case-control study suggested that a phytoestrogen-rich diet may offer protective benefits, by demonstrating an inverse relationship between the odds-adjusted risk for breast cancer and urinary phytoestrogen excretion (18). Interest in phytoestrogens as natural anticancer agents was stimulated by animal studies using the classical animal model of chemically induced breast cancer. In this model, soy containing isoflavones was found to reduce tumor formation significantly in a dose-dependent manner (19). These animal studies are supported by numerous in vitro studies that have shown that daidzein and genistein can inhibit cell growth (20,21). Genistein (5,7,4′-trihydroxyisoflavone) and daidzein (7,4′-dihydroxyisoflavone), the two major isoflavonoids in soy, have antiproliferative effects on the growth of human...
breast cancer cells in culture and were candidates for use in the prevention of breast cancer (19, 22). Genistein and daidzein are structurally similar to 17β-estradiol, thus their identification as phytoestrogens. Because these molecules are similar to estradiol, they were initially considered to have estrogen agonist/antagonist activity (23). However, other mechanisms have been proposed, including PTK inhibition, topoisomerase II inhibition, induction of differentiation, and inhibition of oxidation events (24, 25).

The mechanism by which soy influences the risk of breast cancer is not known. Therefore, we investigated the effects of genistein and daidzein on the BRCA2 tumor suppressor gene and then their mode of action.

Materials and Methods

Cell Culture and Treatments

The breast cancer cell lines MCF7 (26) and MDA-MB-231 (27) and the normal mammary cell line MCF10a (28) were obtained from the American Type Culture Collection (ATCC). MCF7 cells were cultured in RPMI 1640 media and supplemented with 2 mM glutamine (Sigma), 1% gentamycin (Sigma), 10% fetal bovine serum (FBS; Life Technologies), and 10 µg/ml insulin (Sigma) in a humidified atmosphere at 37°C containing 5% CO2. MCF10a cells were maintained in DMEM/Ham’s F-12 containing 5% horse serum (Life Technologies), 2 mM glutamine, 1% gentamycin, 10 mg/ml insulin, 20 ng/ml epidermal growth factor (EGF; Sigma), 100 ng/ml cholera toxin (Sigma), and 0.5 µg/ml hydrocortisone (Sigma) at 37°C with 5% CO2. MDA-MB-231 cells were grown in Leibovitch 15 media, with 2 mM glutamine, 1% gentamycin, and 15% FBS, in a 37°C humidified atmosphere without CO2.

All these media contained Phenol Red, which includes a small quantity of estrogens. Our quantification of mRNA and protein are all normalized to untreated cells and are thus not perturbed by the negligible and constant presence of Phenol Red.

The estrogen receptor (ER) status was confirmed by immunocytochemistry. MCF7 were ER α+/β+, MCF10a ER α−/β−, and MDA-MB-231 ER α−/β+.

Effect of Phytoestrogen Treatments on Cell Proliferation

Cells (1 x 10⁶ per T75 flask) were seeded in medium and treated with increasing doses (2.5, 5, 10, 20, 30, or 40 µg/ml) of genistein (Sigma) or (10, 20, 30, 40, 50, or 60 µg/ml) of daidzein (Sigma) diluted in DMSO. After 72 h, cells were trypsinized and the cell numbers scored on a Malassez cell using Trypan Blue to determine the IC50 dose.

Metabolic Labeling

After 3 days of incubation with or without isoflavone, the medium was changed and cells were labeled with 100 µCi of 35S-methionine (1,000 Ci/mM; Amersham International PLC, UK) for 24 h. Metabolic labeling was stopped by adding 10 ml cold PBS and cells were gently washed twice with PBS at 4°C.

Protein Extraction

After washing in PBS, the cells were solubilized in the flask with 5 ml lysis buffer [150 µM NaCl, 0.02 M Tris-HCl (pH 8.0), 1% Nonidet P40, 500 µM EDTA, 10% glycerol, 1% sodium orthovanadate, 1 M NaF, with protease inhibitor cocktail tablet (100 µM PMSF, 2 mg/ml leupeptin, 2 mg/ml pepstatin)] at 4°C for 30 min, followed by ultracentrifugation at 30,000 g for 30 min. The supernatant was collected for analysis.

Affinity Chromatography

BRCA2 protein was purified and quantified by two successive affinity chromatographies using a Biocad Sprint System (Applied Biosystems). First, a POROS 20 HE (heparin) media column (4.6 mmD/50 mm; Perseptive Biosystems) was used to purify the totality of DNA-binding proteins, including BRCA1 and BRCA2 (29, 30), after elution with a gradient of NaCl from 0.1 to 1 M in 20 mM MES at pH 5.5. The flow rate was 5 ml/min and, after detection of proteins by UV at 280 nm, 0.5 ml fractions were collected. Ten microliters of each fraction was counted in 5 ml scintillation liquid. Fractions containing radioactivity were pooled.

Radiolabeled BRCA2 was then immunoprecipitated with 5 µg of anti-BRCA2 antibodies (556 448, BD PharMingen, which recognizes an epitope between amino acids 2,586 and 2,600, or 66066E, BD PharMingen, which recognizes an epitope between amino acids 1,323 and 1,346) at 37°C for 1 h. A POROS A column (4.6 mmD/50 mm; PerSeptive Biosystems, Inc.) was used to isolate the immunoprecipitate by elution with 0.1% (v/v) 12 mM HCl/0.15 M NaCl at pH 2. The flow rate and protein analysis were the same as above, except 1 ml fractions were collected and the radioactivity was measured on the whole fraction in 5 ml scintillation cocktail.

The relative amount of BRCA2 protein was expressed as a ratio as follows: 100× [disintegrations per minute (dpm) bound to the anti-BRCA2 antibodies/dpm purified on heparin column]. All data were expressed as means (standard deviation of eight assays); Student’s t-test was used for the statistical analysis.

RNA Isolation

After 72 h, at 70–80% confluency, cells were extracted and total RNA was isolated using RNA B™ reagent (Bioprobe Systems) according to the manufacturer’s protocol. RNA was quantified by spectrophotometry (260/280 nm).
Reverse Transcription

RNA was reverse transcribed in a final volume of 15 μl containing 0.2 μg/ml of random hexamers, 200 mM DTT, 1X bulk First Strand Mix, and 5 μg RNA. The RNA and DEPC water were first incubated at 65°C for 10 min, and then the random primer, bulk buffer, and DTT were added and the samples incubated at 37°C for 1 h. Reverse transcriptase was inactivated by heating at 95°C for 5 min.

Real-Time PCR

All reactions were performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) 96-well thermal cycler, which detects the signal from a fluorogenic probe during PCR. Quantitative PCR was performed with the TaqMan PCR Core Reagent Kit (Applied Biosystems). The results incorporate two independent RNA extractions (E1 and E2) and three reverse transcriptions, two for the first extraction (E1) and an one for the second extraction (E2). BRCA2 primers and probe were chosen with Primer Express Software (Applied Biosystems). The sequence of the probe was 5′-9818 ACTGTAATCTCCGGGCCTAGACTTGCTCAAA 9847-3′, the forward primer was 5′-9794 CCAAGTGGTCCCACCACCACCCACCACACCC ACC9812-3′, and the reverse primer was 5′-9895 CACAAATTAGGAGAGCACATCGAAGC9870-3′.

Multiplex PCR was performed using an endogenous control (18S rRNA) provided by Applied Biosystems and used according to the manufacturer’s conditions. Adequate quenching was observed for probes with the reporter (VIC for 18S and FAM for BRCA2) at the 5′ end and the quencher (TAMRA) at the 3′ end.

The ABI Prism 7700 Sequence Detection systems consists of four major elements: 1) uniquely designed fluorogenic oligonucleotide probes, 2) a PCR assay that exploits the 5′ nuclease activity of Taq DNA polymerase, with its polymerization-dependent cleavage of the target-specific probe to detect the accumulation of specific PCR product, 3) instrumentation that measures the fluorescent signal from 96 samples during the thermal cycle reaction, and 4) software that processes and analyzes the fluorescence data.

Analysis with the comparative C_T (threshold cycle parameter) method is similar to the standard curve method, except it uses arithmetic formulas for relative quantification. The amount of target is normalized to the co-amplified 18S reference and compared with a reference sample with the equation 2^-ΔΔCT. For the ΔΔ C_T calculation to be valid, the efficiency of the target amplification and the efficiency of the reference amplification must be approximately equal.

Treatment With an Anti-Estrogen

MCF7 (ER α+/β+) and MDA-MB-231 (ER α–/β+) cells were treated with 50 μM Yp537 (31) for 30 min to inhibit estrogen receptor dimerization and then incubated with genistein for 1 h.

Regulation of BRCA2 Expression by Actinomycin D or Cycloheximide

The effects of actinomycin D and cycloheximide were only studied in cell lines where BRCA2 mRNA variation was observed. In MDA-MB-231 and MCF10a treated for 72 h with 5 μg/ml genistein, translational regulation was studied by adding 10 μg/ml cycloheximide, and transcriptional regulation was studied by adding 2 μg/ml actinomycin D for the last 6 h of the 72-h incubation of cells with or without genistein. After actinomycin D or cycloheximide treatment, BRCA2 mRNA was extracted and quantified by real-time reverse transcriptase-polymerase chain reaction (RT-PCR).

Results

Cell Proliferation Assay

A dose-effect curve was carried out for each phytoestrogen. The concentration leading to 50% inhibition of the proliferation (IC_{50}) was selected for further treatments: 5 μg/ml genistein or 20 μg/ml daidzein; results are plotted in Fig 1. Control treatment with DMSO, used to dissolve genistein and daidzein, had no effect on proliferation.

The circled numbers correspond to the concentration of 50% inhibition of proliferation (IC_{50}) for each cell line.

Quantification of BRCA2 Protein After Treatment With Genistein and Daidzein

The effects of genistein and daidzein on BRCA2 expression were studied in two human mammary tumor cell lines, MCF7 (ER α+/β+) and MDA-MB-231 (ER α–/β+), and one normal human breast epithelial cell line, MCF10a (ER α–/β–). The quantification of BRCA2 protein was performed by two successive affinity chromatographies as described above. No variation in BRCA2 protein was found after genistein or daidzein treatment, as determined by immunoaffinity to two anti-BRCA2 antibodies (Fig. 2).

Quantification of BRCA2 mRNA

BRCA2 mRNA from cells treated with daidzein or genistein was quantified by real-time RT-PCR. BRCA2 was significantly increased in MCF10a (ER α–/β–) and MDA-MB-231 (ER α–/β+) but remained constant in MCF7 (ER α+/β+) after treatment with genistein (Fig. 3) by comparison with untreated cells normalized to one with the comparative C_T method. Daidzein had no effect on BRCA2 in any of the three cell lines.

Phytoestrogen and Estrogen Receptor Pathway

We used an anti-estrogen, Yp537, which is known to block receptor dimerization, in the MCF7 (ER α+/β+) and MDA-MB-231 (ER α–/β+) cell lines. After 30-min incubation with Yp537 followed by 1 h incubation with genistein, no difference was observed between the MCF7 (ER α+/β+) and MCF10a (ER α–/β–) cell lines, as shown in Fig. 4. This result suggests that the effects of genistein are likely mediated through other pathways.
treated with both drugs or with genistein alone (Fig. 4A). In MDA-MB-231 (ER $\alpha$–/$\beta$+), an induction with genistein and Yp537 was obtained by comparison with MDA-MB-231 with genistein alone (Fig. 4B). These results suggested that genistein does not act through the ER pathway, neither ER $\alpha$ nor ER $\beta$.

**BRCA2 mRNA Regulation by Use of Actinomycin D or Cycloheximide**

That *BRCA2* mRNA levels did not correspond to *BRCA2* protein abundance in MCF10a and MDA-MB-231 after genistein treatment might be explained by transcriptional and/or posttranslational regulation, so we studied *BRCA2* mRNA regulation by use of actinomycin D and cycloheximide. To determine whether genistein induced *BRCA2* accumulation by altering its synthesis, we measured *BRCA2* mRNA level in MCF10a and MDA-MB-231 treated for 72 h with genistein and with actinomycin D added for the last 6 h. In both cell lines, we found a decrease in mRNA when the cells were treated with both genistein and actinomycin D by comparison with cells treated with genistein or actinomycin D alone (Fig. 5A and B). This demonstrated that genistein has a positive action on the transcriptional level of *BRCA2* mRNA in MDA-MB-231 or MCF10a.

To determine the effects of genistein on the level of de novo *BRCA2* protein synthesis, MCF10a and MDA-MB-231 were treated with genistein for 72 h, with cycloheximide added for the last 6 h to block protein synthesis. In MDA-MB-231 cells, we observed the same decrease
in BRCA2 in cells treated with cycloheximide as treated with genistein and cycloheximide (Fig. 6A). In MCF10a, cycloheximide significantly decreased BRCA2 mRNA, although an equivalent decrease was not found in MCF10a treated with genistein and cycloheximide (Fig. 6B). These results suggest that genistein induces de novo protein synthesis in MCF10a but not in MDA-MB-231.

Discussion

The purpose of this study was to determine whether phytoestrogens such as daidzein and genistein, the main components of soy, might affect the expression of the BRCA2 tumor suppressor gene in sporadic breast cancer.

The quantification of BRCA2 mRNA by real-time quantitative RT-PCR allowed us to compare the effect of two phytoestrogens (daidzein and genistein) in comparison with untreated cells, which were normalized to 1. Genistein increased BRCA2 mRNA in MDA-MB-231 (ER α–/β+) and MCF10a (ER α–/β–) cells. Daidzein was without effect on three breast cell lines.

BRCA2 protein was measured by perfusion affinity chromatography (32). Two different polyclonal anti-BRCA2 antibodies were used, 66066E and 556 448. The 66066E antibody was raised against a sequence in the middle of the BRCA2 protein, whereas the 556 448 antibody was raised in BRCA2 in cells treated with cycloheximide as treated with genistein and cycloheximide (Fig. 6A). In MCF10a, cycloheximide significantly decreased BRCA2 mRNA, although an equivalent decrease was not found in MCF10a treated with genistein and cycloheximide (Fig. 6B). These results suggest that genistein induces de novo protein synthesis in MCF10a but not in MDA-MB-231.

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Figure 3. Effect of 20 µg/ml daidzein and 5 µg/ml genistein on the level of BRCA2 mRNA in MCF7, MCF10a, and MDA-MB-231, normalized to untreated cells using real-time quantitative RT-PCR. Each measure was performed on two extractions and three RTs and is expressed as means ± SD. Statistic analysis was performed using Student’s t-test (*, P < 0.05).

Figure 4. Effect of 30-min treatment with 50 µM Yp537, followed by 5 µg/ml genistein for 1 h in MCF7 (A) and MDA-MB-231 (B). mRNA was extracted and quantified by real-time RT-PCR. Control cells and cells treated with genistein were normalized to 1.
against the C-terminal portion. Neither genistein nor daidzein had any effect on the level of BRCA2 protein in MDA-MB-231, MCF7, or MCF10a. The specificity of the anti-BRCA2 antibodies was demonstrated previously by peptide competition assays (33). For that, we used the same 20 amino acid synthetic peptides used for immunization. Complete competition was obtained with 66066E (unpublished data) and 556 448.

A possible explanation for the disparity observed between the levels of BRCA2 protein and mRNA after genistein treatment is that BRCA2 might be posttranscriptionally and/or translationally regulated, resulting in no variation in the protein level even though the mRNA was increased in MCF10a and MDA-MB-231 after treatment. This was tested using actinomycin D, which binds to DNA and inhibits transcription; we found that genistein increased the transcription of BRCA2 in MDA-MB-231 and MCF10a. Regulation at the level of mRNA translation was tested by using cycloheximide. We found that amount of BRCA2 mRNA was not blocked by cycloheximide, indicating that de novo protein synthesis was not required in MDA-MB-231, although de novo protein synthesis seemed necessary in MCF10a after treatment with genistein.

Others who have found that the BRCA1 mRNA increases in response to phytoestrogens while the protein level remains unchanged have suggested that BRCA1 degradation might be due to cathepsin-like protease and not to the proteasome (34). However, nothing is yet known about the degradation of BRCA2, and this needs further investigation.

Estrogens may modulate tumor suppressor gene transcription in a hormone-dependent manner by increasing BRCA1 mRNA expression by 17-fold and BRCA2 mRNA expression by approximately by 50-fold in human breast cancer cell lines such as MCF7 (35). We therefore investigated the effects of genistein and daidzein, which have structures similar to 17β-estradiol, on MCF7, MDA-MB-231, and MCF10a, three breast cell lines with different estrogen receptor status. The binding affinity of genistein to ER is comparable with that of 17β-estradiol (36), but daidzein lacks one hydroxyl group and has a much lower binding affinity, which may explain why only genistein increased BRCA2 mRNA.

Figure 5. Effect of actinomycin D (Act D) on BRCA2 mRNA expression. MDA-MB-231 (A) and MCF10a (B) were incubated for 72 h with 5 μg/ml genistein and with or without 2 μg/ml Act D for the last 6 h. mRNA was extracted and quantified by real-time RT-PCR. BRCA2 in untreated and genistein-treated cells was normalized to 1. Statistical analysis were performed using Student’s t-test (**, P < 0.05).
Competition experiments have shown that phytoestrogens such as genistein and daidzein have higher affinity for ER\textsubscript{β} than for ER\textsubscript{α} (37), and it has been shown that ER\textsubscript{α} and ER\textsubscript{β} differ in their ability to activate gene expression (38). Phytoestrogens are believed to exert their chemopreventive action by interacting with estrogen receptors, although alternative mechanisms, most notably inhibition of protein kinase activity, have been proposed (39). The phytoestrogen responses mediated by ER\textsubscript{α} and ER\textsubscript{β} may vary with different composition of their coactivators, which act at the promoters of target genes such as BRCA2.

To determine the level of action of genistein, we used Yp537, an anti-estrogen. With or without Yp537, genistein had no effect on the level of BRCA2 mRNA in MCF7 (ER\textsubscript{α+}/\textsubscript{β+}), whereas in MDA-MD-231 (ER\textsubscript{α−}/\textsubscript{β+}) and MCF10a (ER\textsubscript{α−}/\textsubscript{β−}) the level of BRCA2 mRNA was increased. These results suggest that genistein does not affect BRCA2 levels via the estrogen receptor pathway. Moreover, MDA-MB-231 was previously described by the ATCC as ER−, but now it is well known that they are ER\textsubscript{α−} and they express ER\textsubscript{β} (40,41).

In conclusion, genistein induced BRCA2 mRNA expression in MCF10a (ER\textsubscript{α−}/\textsubscript{β−}) and MDA-MB-231 (ER\textsubscript{α−}/\textsubscript{β+}) breast cell lines. No variation of BRCA2 protein level was observed. This effect is not mediated via the ER\textsubscript{α} nor the ER\textsubscript{β} pathway. Further investigations are necessary to identify the mechanism by which genistein affects BRCA2 gene expression.

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