

Antiproliferative activity of daidzein and genistein may be related to ER α /c-erbB-2 expression in human breast cancer cells

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Abstract. In this study, we investigated the antiproliferative activity of the isoflavones daidzein and genistein in three breast cancer cell lines with different patterns of estrogen receptor (ER) and c-erbB-2 protein expression (ER α -positive MCF-7 cells, c-erbB-2-positive SK-BR-3 cells and ER α /c-erbB-2-positive ZR-75-1). After treatment at various concentrations (1-200 μ M for 72 h), the effect of daidzein and genistein on the proliferation of different cell types varied; these effects were found to be associated with ER α and c-erbB-2 expression. Daidzein and genistein exhibited biphasic effects (stimulatory or inhibitory) on proliferation and ER α expression in MCF-7 cells. Although 1 μ M daidzein significantly stimulated cell growth, ER α expression was unaffected. However, genistein showed marked increases in proliferation and ER α expression after exposure to <10 μ M genistein. Notably, the inhibition of cell proliferation by 200 μ M genistein was greater compared to that by daidzein at the same concentration. Daidzein and genistein significantly inhibited proliferation of SK-BR-3 and ZR-75-1 cells in a dose-dependent manner. In addition, ER α and c-erbB-2 expression was reduced by daidzein and genistein in both SK-BR-3 and ZR-75-1 cells in a dose-dependent manner. However, the effect of genistein was greater compared to that of daidzein. In conclusion, the isoflavones daidzein and genistein showed anti-breast cancer activity, which was associated with expression of the ER α and c-erbB-2 receptors.

Introduction

Isoflavones are a flavonoid subclass that includes daidzein (7,4-dihydroxyisoflavone) and genistein (4',5,7-trihydroxyisoflavone), which are the predominant isoflavones in legumes, such as soybeans, and are commonly found in a variety of human foods (1,2). Several epidemiological studies have

revealed an inverse association between isoflavone consumption and the risk of breast cancer in Asians, indicating that dietary intervention may reduce the risk of breast cancer (3-5).

Based on this hypothesis, a number of studies have investigated the pharmacological activities of isoflavones as candidate anticancer agents. Many have reported that isoflavones significantly inhibit breast cancer cell growth *in vitro* (6-8), and that this growth inhibition stimulates the signal transduction pathway leading to apoptosis. Moreover, our previous studies support the theory that isoflavones, including genistein and daidzein, exhibit anticancer activity against breast cancer both *in vivo* (9,10) and *in vitro* (11,12).

Breast cancer is one of the cancers most frequently diagnosed in women. According to a US National Cancer Institute (NCI) report, one in eight women in the US (~13.3%) will develop breast cancer during their lifetime (13,14). Breast cancer is a group of heterogeneous diseases that manifest in several clinical, molecular and histopathological forms; this makes effective chemotherapy difficult. The response to a cancer drug may differ according to biological factors including estrogen receptor (ER) status, progesterone receptor status and c-erbB-2 expression. The present study compared the anticancer activity of daidzein and genistein via their effect on ER α and c-erbB-2 expression in breast cancer cell lines.

Materials and methods

Cell culture. Human breast cancer MCF-7, SK-BR-3 and ZR-75-1 cell lines were purchased from the KCLB (Korean Cell Line Bank, Seoul, Korea) and were used in the present study. Each cell line was routinely maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (50 U/ml penicillin and 50 μ g/ml streptomycin; Gibco, Carlsbad, CA, USA) at 37°C in a humidified atmosphere containing 5% CO₂. Each cell type was plated at a density of 0.8-1.2x10⁶ cells/well and 2.5-5.0x10³ cells/well on 6- and 96-well plates for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and immunoblotting assays, respectively. Daidzein and genistein were purchased from LC Laboratories® (Woburn, MA, USA) and dissolved in dimethyl sulfoxide (DMSO; final concentration 0.1% in medium).

MTT assay. Each cell type was exposed to daidzein or genistein at 1, 10, 100 and 200 μ M for 72 h. Following treatment,

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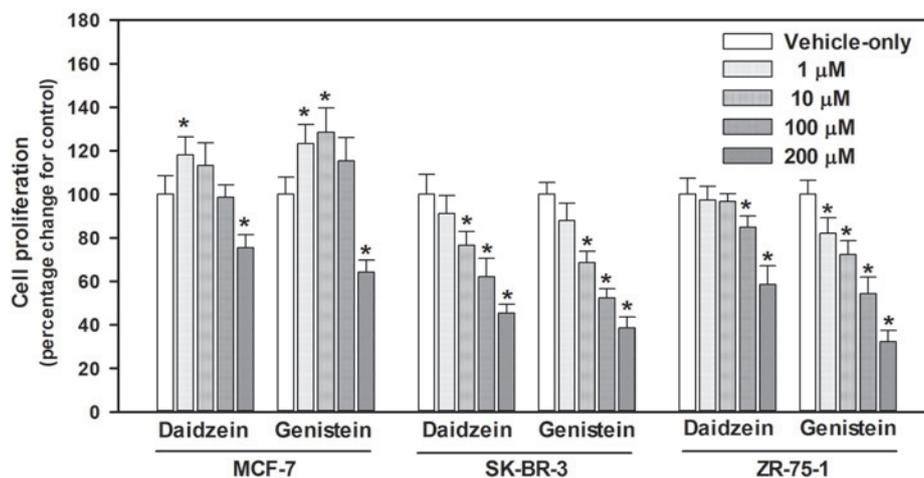


Figure 1. Antiproliferative effects of daidzein and genistein on MCF-7, SK-BR-3 and ZR-75-1 cells. Each cell line was exposed to daidzein or genistein at 1, 10, 100 and 200 μ M for 72 h. The data are presented as the percentage change compared with the vehicle-only group. * P <0.05, significantly different from the vehicle-only group.

the plated cells were incubated with MTT (0.5 mg/ml final concentration) for 4 h at 37°C. After discarding the medium from the plates, 100 μ l DMSO was added to each well. The plates were placed for 5 min at room temperature with agitation, so that complete dissolution of formazan was achieved. The absorbance of the MTT formazan was determined at 540 nm by a UV/VIS spectrophotometric plate reader (Emax; Molecular Devices, Silicon Valley, CA, USA).

Immunoblotting assay. Each cell type was exposed to daidzein or genistein at 1, 10, 100 and 200 μ M for 72 h. Cells were lysed in RIPA buffer (1% NP-40, 150 mM NaCl, 0.05% DOC, 1% SDS, 50 mM Tris, pH 7.5) containing protease inhibitor for 1 h at 4°C. The supernatant was separated by centrifugation and the protein concentration was determined by Bradford protein assay kit II (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were transferred onto nitrocellulose membranes (0.45 μ m). The membranes were blocked with a 1% bovine serum albumin (BSA) solution for 1.5 h and washed twice with phosphate-buffered saline (PBS) containing 0.2% Tween-20 and incubated with the respective primary antibodies (ER α , C-erbB-2, β -actin; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C. The next day, the immunoreaction was continued with the secondary rabbit anti-rabbit horseradish peroxidase-conjugated antibody after washing for 2 h at room temperature. The specific protein bands were detected using an Opti-4CN Substrate kit (Bio-Rad Laboratories).

Apoptosis detection. Apoptotic morphological changes were determined by DAPI (4',6-diamidino-2-phenyl-indole) staining on ZR-75-1 cells exposed to daidzein or genistein at a concentration of 100 μ M for 72 h. After harvesting the cells exposed to daidzein or genistein, the cells were seeded onto poly-L-lysine-coated slides and fixed with 4% methanol-free formaldehyde solution for 30 min. Mounting medium with DAPI (Molecular Probes, Invitrogen) was then dispersed over the entire section of slides. Mounted slides were stored at 4°C without light. Each slide was observed under an Axio vision 4.0 fluorescence microscope (Carl Zeiss Inc., New York, NY, USA).

Statistical analysis. The values are expressed as the means \pm standard deviation (SD). Data were analyzed by unpaired Student's t-test or one-way analysis of variance followed by Dunnett's multiple comparison test (SigmaStat, San Jose, CA, USA). P <0.05 was considered to indicate a statistically significant difference.

Results

Antiproliferative activities. We compared the antiproliferative effects of daidzein and genistein on MCF-7, SK-BR-3 and ZR-75-1 cells (Fig. 1). Exposure of MCF-7 cells to various concentrations (1, 10, 100 and 200 μ M) of daidzein and genistein for 72 h resulted in similar biphasic antiproliferative effects. Although daidzein and genistein at higher concentrations (200 μ M) inhibited proliferation by 24.64 and 35.79%, respectively, compared with the control, both compounds stimulated cell growth at lower concentrations (<10 μ M). Daidzein and genistein (1 and 10 μ M, respectively) induced cell growth by ~18 and 28%, respectively.

The two compounds greatly inhibited growth of SK-BR-3 cells in a dose-dependent manner. Statistically significant inhibition of cell proliferation was first observed in cells treated with 10 μ M daidzein and genistein (23.46 and 35.79% inhibition, respectively). The IC₅₀ values for daidzein and genistein were 211.70 and 138.13 μ M, respectively.

In ZR-75-1 cells, the antiproliferative effects of genistein were stronger than those of daidzein under the same conditions. Genistein significantly inhibited proliferation of ZR-75-1 cells in a dose-dependent manner; the effect was similar to that of genistein on SK-BR-3 cells. Daidzein significantly inhibited cell growth at 100 and 200 μ M, but not at concentrations <10 μ M. Inhibition of proliferation by higher daidzein concentrations was decreased by 15-41% compared with control levels.

ER α and c-erbB-2 expression. ER α and c-erbB-2 expression was determined in the three cell lines (Fig. 2A). ER α -positive MCF-7 cells exhibited a basal level of c-erbB-2, while

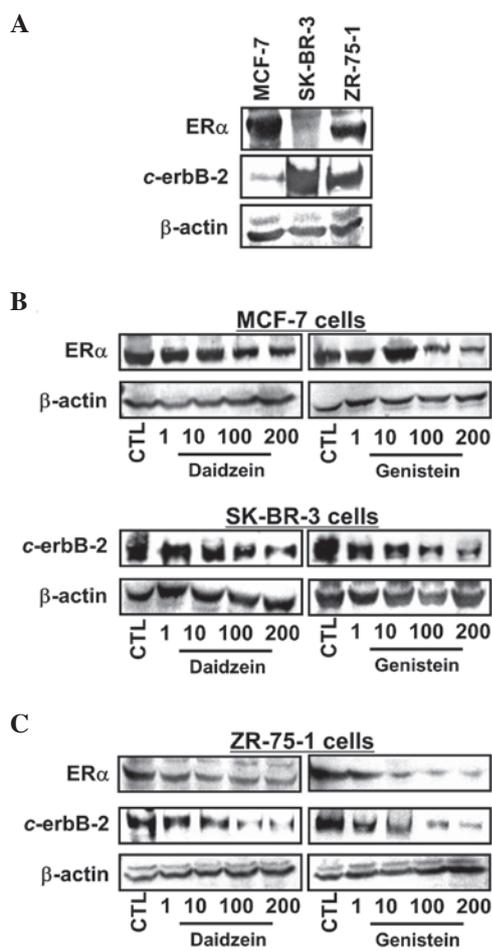


Figure 2. Modulation of ER α and c-erbB-2 expression by daidzein and genistein. (A) ER α and c-erbB-2 expression according to cell type; (B) ER α or c-erbB-2 expression in MCF-7 and SK-BR-3 cells exposed to daidzein or genistein at 1, 10, 100 and 200 μ M for 72 h; (C) ER α or c-erbB-2 expression in ZR-75-1 cells exposed to daidzein or genistein under identical conditions.

SK-BR-3 cells overexpressed c-erbB-2. In ZR-75-1 cells, expression of ER α and c-erbB-2 was observed. As presented in Fig. 2B and C, when cells were exposed to 1, 10, 100 and 200 μ M daidzein and genistein for 72 h, ER α expression by MCF-7 cells exposed to genistein showed a pattern similar to the antiproliferative effects and no stimulation by lower daidzein concentrations was detected. However, both daidzein and genistein reduced ER α expression at higher concentrations (100 and 200 μ M). Notably, ER α expression in ZR-75-1 cells was downregulated by daidzein and genistein at the concentrations used; again, the effect of genistein was greater. c-erbB-2 expression was decreased by daidzein and genistein in a dose-dependent manner in SK-BR-3 and ZR-75-1 cells, regardless of their basal c-erbB-2 levels.

Apoptosis induction. DAPI staining was used to detect apoptotic changes after exposing ZR-75-1 cells to either daidzein or genistein at a concentration of 100 μ M for 72 h (Fig. 3). Apoptotic morphological features, such as cell shrinkage and dot-shaped nuclear fragments, were observed following exposure of cells to daidzein and genistein. Compared with control cells, exposure to genistein resulted in a marked increase in apoptotic morphological features.

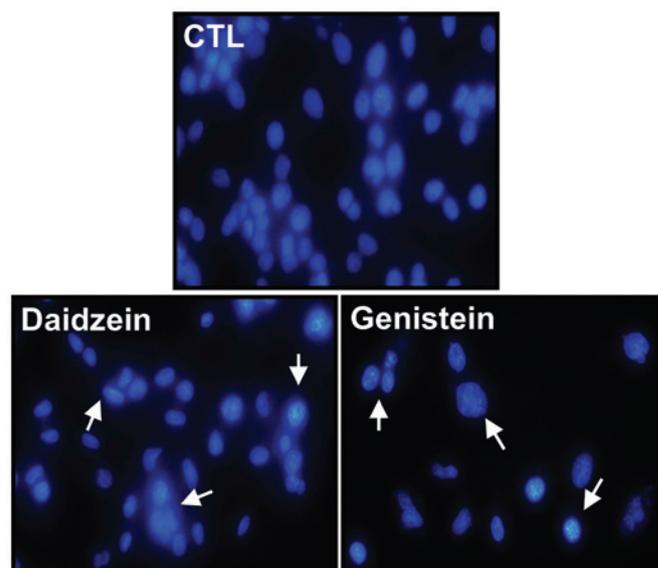


Figure 3. Apoptotic morphological changes by daidzein and genistein. ZR-75-1 cells exposed to daidzein or genistein at 100 μ M for 72 h. Arrows indicate apoptotic features.

Discussion

In this study, we investigated the antiproliferative effects of the isoflavones daidzein and genistein in the following three breast cancer cell lines: MCF-7 (ER α ⁺), SK-BR-3 (c-erbB-2⁺⁺) and ZR-75-1 (ER α ⁺ and c-erbB-2⁺).

The effects differed greatly according to breast cancer cell type. Daidzein and genistein exhibited a significant antiproliferative effect against the three cell lines, however, the effect on MCF-7 cells was biphasic (inhibitory at high concentrations and stimulatory at low concentrations). This is consistent with previous studies that revealed isoflavones exert a biphasic effect on cell proliferation (15-17), which may be explained by their estrogen-like activity. Isoflavones are known as phytoestrogens, which are molecules structurally similar to 17 β -estradiol (18). This characteristic may cause not only ER-agonistic but also ER-antagonistic effects and was likely responsible for the biphasic effect on MCF-7 cell proliferation. This mechanism may prove useful in the prevention of ER-positive breast cancer. ER α is a member of the steroid receptor superfamily and regulates growth, differentiation and other processes in various target cells by regulating transcriptional processes (19). It is also important in the development and progression of breast cancer (20) and is expressed in ~70% of breast cancers (21), which makes it difficult to obtain a response to cancer drug treatment.

By contrast, daidzein and genistein significantly inhibited the growth of SK-BR-3 and ZR-75-1 cells in a dose-dependent manner under the same conditions; however, the antiproliferative effects of daidzein were relatively weak. Genistein had a stronger effect on ER α -positive SK-BR-3 cells than ER α - and c-erbB-2-positive ZR-75-1 cells; the IC₅₀ values were 138.13 and 127.71 μ M in SK-BR-3 and ZR-75-1 cells, respectively. c-erbB-2 (HER2/neu) encodes a 185-kDa transmembrane glycoprotein that belongs to the epidermal growth factor receptor family of type I receptor tyrosine kinases (ErbB family). c-erbB-2 overexpression has been reported in ~25-30% of human breast

cancers (22,23). Overall, genistein exhibited an effect on the three cell lines; the gap between stimulation and inhibition of proliferation of MCF-7 cells exposed to genistein was larger than that of daidzein and the antiproliferative effects on the other cell lines were more marked. This may be due to the estrogenic activity of genistein, which has a stronger affinity for ERs than daidzein (12).

Moreover, when cell lines were exposed to daidzein and genistein under the same conditions, the modulation of ER α and c-erbB-2 expression by daidzein and genistein reflected their pattern of antiproliferative activity. Induction of ER α expression in MCF-7 cells exposed to <10 μ M daidzein was unclear; at higher concentrations, daidzein downregulated the ER α expression. ER expression in ZR-75-1 cells was also slightly reduced by daidzein, regardless of dose. Similar to the pattern of MCF-7 cell proliferation by genistein, its application at a lower concentration stimulated ER α expression; by contrast, genistein at a higher concentration markedly reduced this expression. This reduction was also observed in ZR-75-1 cells exposed to genistein, even at a lower concentration (1 μ M).

c-erbB-2 expression by SK-BR-3 and ZR-75-1 cells was decreased by daidzein or genistein, with the latter having a greater effect. It has been reported that the mechanism underlying the anticancer activity of genistein and daidzein involves downregulation of c-erbB-2 (24,25) and enhanced ER-erbB-2 cross-talk in breast cancer cells (26).

The isoflavones daidzein and genistein induced apoptosis as evidenced by the apoptotic morphological features that were observed in cells exposed to daidzein and genistein. Several studies have indicated that anticancer drugs or cancer chemopreventive agents act through the induction of apoptosis in various cancer cells (27,28). Recently, the regulation of apoptosis has been proposed as a promising target for cancer chemotherapy. Our data suggest that daidzein and genistein may possess anticancer properties.

Based on our results, although both daidzein and genistein may be deleterious at lower concentrations by stimulating proliferation of MCF-7 cells, genistein may offset those effects and could be beneficial for breast cancer prevention under certain conditions. Genistein at higher concentrations had a marked anti-estrogenic effect via ER α -downregulation and the growth-promotion effect of genistein was attenuated in ER α and c-erbB-2-coexpressing ZR-75-1 cells via downregulation of c-erbB-2.

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