Induction of human adenocarcinoma cell differentiation by the phytoestrogen genistein is independent of its antiestrogenic function

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Abstract. The objective of the present study was to determine if genistein can induce human breast adenocarcinoma cell maturation. To gain understanding on its mechanism of action, we used estrogen receptor-positive (ER+) MCF-7, and ER- MDA-MB-468 cells. Treating these cells with genistein resulted in growth inhibition accompanied by increased cell maturation, which was evaluated by the production of caseins and lipids. These maturation markers were optimally expressed after nine days of treatment with 30 μM of genistein. Since both ER+ and ER- cells became differentiated, we conclude that the ER is not a component of the genistein-initiated scheme of cellular differentiation.

Introduction

Treatment of preneoplastic or neoplastic cells with natural or synthetic agents that induce terminal differentiation at nontoxic concentrations may find applications in cancer prevention or treatment. Under the influence of such agents, proliferating tumor cells enter the program of terminal differentiation, cease dividing, and consequently lose their tumorigenic potential (1). This approach, known as differentiation therapy, produced promising antitumor agents with no or low toxicity and is now gaining popularity among cancer researchers (2).

We previously identified the soybean isoflavone genistein as a potent inducer of cell differentiation in a variety of tumor cell lines (3-5).

The main source of genistein in the human diet are soybeans and soy-products (tofu, soy-milk, soy-flour etc.). Following their consumption, genistein is generated through hydrolysis, by intestinal flora of β-glucoside (known as genistin), which is the most dominant natural form in soybeans. The resulting unconjugated isoflavone (genistein) is absorbed from the gastrointestinal tract, and, when it reaches the liver, it becomes conjugated by glucuronosyl-transferases and sulfotransferases (6). The plasma and urine of consumers of soy-rich diets contain up to an estimated 3.3 μM of genistein and its metabolites (7-10).

Genistein was identified initially as a phytoestrogen (11) shown to compete with estradiol in MCF-7 cells for the estrogen receptor and to elucidate other typical estrogen-induced responses (12). Subsequently, genistein was identified as a specific protein tyrosine kinase inhibitor (13,14) and a topoisomerase (topo) II inhibitor (3,15,16). When introduced at relatively high concentrations in cultures of T-cell leukemia and other tumor cell lines, genistein delayed cell-cycle progression and induced apoptosis (17-20).

Although genistein has been studied extensively as a cancer preventive agent against mammary carcinogenesis (reviewed in refs. 21,22), its effects on the differentiation of human breast adenocarcinoma cells have not been examined as of today. The experiments presented below were designed to fill the above gap and also to evaluate the possible involvement of the estrogen receptor during the scheme of phytoestrogen-induced cell differentiation.

Materials and methods

Chemicals and reagents. Genistein (4',5,7-trihydroxy- isoflavone) and daidzein (4',7-dihydroxyisoflavone) were purchased from Indofine Chemical Company (Somerville, NJ); anti-human casein was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY), and Red Lipid from Rowley Biochemical Institute (Rowley, MA).

Cells and culture conditions. The MDA-MB-468 and MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD). MDA-MB-468 cells were cultured in MEM medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and glycine. MCF-7 cells were cultured in RPMI-1640 medium supplemented with 15% fetal bovine serum, penicillin (100 units/ml), and glycine. All cells were plated at 5x10⁴ in 1 ml medium and cultured at 37°C in a humidified atmosphere of 8% CO₂ in air. Treatment with genistein was 24 h later at specified doses. Cell numbers were determined by hemocytometer chamber counting, and viability was monitored by trypan blue exclusion. For immunohistochemistry, cells were cultured in dishes containing cover slips and processed after six or nine days.

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**Immunohistochemical staining.** The presence of casein was determined by immunohistochemical staining using mouse monoclonal antibody essentially as described by Bacus et al. (23). Cells cultured on cover slips were rinsed with phosphate-buffered saline at room temperature and fixed in a quick dip of -20°C methanol-acetone solution; after blocking with milk (5%) for 10 min, the cells were incubated with anti-casein antibody (1:200) overnight at 4°C. Slides were then rinsed with PBS and incubated for 15 min with secondary antibody (biotinylated anti-mouse or anti-rabbit antibodies from Dako Corporation, Carpinteria, CA). The slides were rinsed in PBS and incubated for 15 min with streptavidin peroxidase conjugate (from Dako Corporation). The cells were again rinsed with PBS and incubated for 10 min with AEC chromogen (from BioGenex Laboratories, San Ramon, CA), and finally counter-stained with hematoxylin.

**Flow cytometry.** Cells were plated at 5×10⁴/ml and 25 h later treated either with DMSO or genistein at 14 or 30 μM. At the end of the incubation period (6 h to 9 days), cells were fixed with 4%,6-diamidine-2-phenylindole in 0.1% citrate solution. Cell-cycle distribution was evaluated, using particle analyzing system (Partec AG, Basel, Switzerland) and a computer program from Phoenix Flow Systems, Inc. (San Diego, CA).

**Results**

The effect of genistein and daidzein (the other major soybean isoflavone) on MCF-7 and MDA-MB-468 cell growth is shown in Fig. 1. Isoflavone concentrations tested ranged from 5 μM-150 μM. At 5 μM, genistein treatment induced a small stimulatory effect on MCF-7 cell growth, but at higher concentrations, there was a dose-dependent growth inhibitory action. The stimulatory effect at low concentrations was observed only on MCF-7 cells, and it may be due to genistein's estrogenic action at low concentrations. Genistein was more efficient in inhibiting MDA-MB-468 cells, because the IC₅₀ for these cells was calculated at 21 μM, compared to 31 μM for MCF-7 cells. Cell viability, determined by trypan blue dye exclusion, remained over 90% at genistein concentrations of up to 45 μM, then it began declining with increasing genistein concentrations (data not shown). Daidzein was not as effective as genistein in inhibiting these two cell lines. At daidzein concentrations of up to 25 μM, there was a very small decrease in cell number when compared to untreated controls.

The effect of genistein in inducing the expression of the mature phenotype in MCF-7 and MDA-MB-468 cells was evaluated using immunohistochemical techniques. In these studies, we monitored the presence of neutral lipid droplets and casein, which are milk components characteristically secreted by differentiated mammary epithelial cells (24).

Less than 5% of the untreated MCF-7 or MDA-MB-468 cells stained positive for casein (Fig. 2, panel A; and Fig. 3, panel A). The percentage of casein-secreting cells increased after treatment with genistein for six or nine days in both cell lines. Although 95% of either cell type stained positive for casein (types β and κ when treated with 15 μM or 30 μM genistein, the intensity of the staining was higher when cells were treated with 30 μM genistein for nine days (Fig. 2, compare panels B and C; and Fig. 3, compare panels B and C).

**Table I. Effect of genistein on MDA-MB-468 cell cycle progression.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phase (%)</th>
<th>0</th>
<th>1/4</th>
<th>1</th>
<th>2</th>
<th>4</th>
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<th>9</th>
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<td>53.2</td>
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<td>39.7</td>
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<td>28.9</td>
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<tr>
<td></td>
<td>G2/M</td>
<td>10.7</td>
<td>15.0</td>
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<td>13.9</td>
<td>16.8</td>
<td>15.2</td>
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<tr>
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<td>47.8</td>
<td>60.7</td>
<td>61.6</td>
<td>56.0</td>
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<tr>
<td></td>
<td>G2/M</td>
<td>15.4</td>
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<td>16.0</td>
<td>18.1</td>
<td>13.3</td>
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aCells were treated with DMSO (which is the genistein solvent) 24 h after plating; only attached cells were processed for flow cytometry; bCells were treated with 30 μM genistein dissolved in DMSO, 24 h after plating; only attached cells were processed for flow cytometry.
Figure 2. Induction of mature phenotype in MCF-7 cells as determined by either reactivity with casein antibody (A-C) or neutral lipid production (D-F). Cells were plated at 5x10^4/ml and the next day treated with genistein or DMSO (control). Treatments were as follows: panels A and D, control cells treated with the solvent (DMSO); panels B and E, cells treated with genistein at 15 μM final concentration; panels C and F, cells treated with genistein at 30 μM final concentration. The red stain in the photomicrographs represents the stained casein visualized with biotinylated goat anti-mouse or anti-rabbit antibodies. Lipid visualization was as described (23). The magnification is x400.

Figure 3. Induction of mature phenotype in MDA-MB-468 cells as determined by either reactivity with casein antibody (A-C), or neutral lipid production (D-F). Cells were plated at 5x10^4/ml and the next day treated with genistein or DMSO (control). Treatments were as follows: panels A and D, control cells treated with the solvent (DMSO); panels B and E, cells treated with genistein at 15 μM final concentration; panels C and F, cells treated with genistein at 30 μM final concentration. The red stain in the photomicrographs represents the stained casein visualized with biotinylated goat anti-mouse or anti-rabbit antibodies. Lipid visualization was as described (23). The magnification is x400.

In the untreated MCF-7 and MDA-MB-468 cells, no obvious lipid droplets could be observed (Fig. 2, panel D; and Fig. 3, panel D). After cells were treated with genistein doses of 15 μM and 30 μM, lipid droplets (orange stain) began to appear at four days post-treatment, but optimum production was after nine days of treatment in both cell lines (Figs. 2 and 3, panels E and F). The number and size of lipid droplets increased with increasing genistein doses of up to 45 μM. The difference in lipid content between cells treated with the high and those treated with the low dose is more in the MDA-MB-468 cells (Fig. 3, compare panels E and F). At 45 μM genistein or higher doses, lipid content decreased, probably due to cytotoxicity. Immunohistochemical changes were accompanied by morphological changes. Genistein-treated cells became flattened, and the cytoplasmic and nuclear areas became larger than in untreated cells (Figs. 2 and 3).

The expression of other maturation markers such as ICAM-1 was also induced in both cell types in response to
genistein treatment (data not shown). Daidzein, which differs by only one -OH group from genistein, was ineffective in inducing the maturation of these cell lines (data not shown).

The effect of genistein on cell-cycle progression was also determined using flow cytometry. Actively growing MCF-7 or MDA-MB-468 cells were treated with 15 and 30 µM genistein, and the distribution through the cell cycle was determined at different time intervals. Results for the control (treated with the solvent DMSO) and 30 µM treatment of MDA-MB-468 cells are shown in Table I. Although some changes in cell-cycle distribution were evident in genistein-treated cells, similar changes were present in the control cells. Also, no differences in cell cycle distribution were evident between the genistein-treated and control MCF-7 cells.

Discussion

The use of nontoxic concentrations of certain agents that promote terminal differentiation of human tumor cells is an appealing strategy in the fight against cancer (1,2). Previous studies have suggested that genistein and daidzein may be the components of soybeans that are active during chemoprevention (21). There are numerous reports of genistein as an effective inducer of cell differentiation in a variety of tumor cells (reviewed in ref. 5). Daidzein, the other major isoflavone of soybeans, has repeatedly been found ineffective as a differentiating agent, although in two isolated reports it has also been shown to induce the mature phenotype in HL-60 and MEL cells (25,26). We report here that genistein induces a mature phenotype in two breast cancer cell lines, as determined by the detection of milk components (casein and lipid droplets) in the cytoplasm, and morphological alterations.

Mechanistically, genistein may induce breast cancer cell differentiation through one of three independent pathways. First, it may inhibit the enzymatic activity of a protein tyrosine kinase that alters the phosphorylation, and subsequently the expression of key regulatory enzymes which may control cell cycle progression (27,28). Second, genistein may bind to the ER and modulate the expression of estrogen responsive genes leading to the expression of the differentiated phenotype. Third, by stabilizing topoisomerase II to DNA, it may produce dynamic changes in the chromatin structure. These changes lead to alterations in gene expression which favor the differentiated phenotype (reviewed in ref. 5).

We previously reported that mammary tissue and tumors from rats injected with genistein did not cause alterations in the patterns of tyrosine phosphopeptides (29). Also, no such differences were evident in MCF-7 and MDA-MB-468 cells following their treatment with genistein concentrations that were sufficient to induce differentiation (data not shown). A genistein concentration of 120 µM was reported to be required for reducing PTK activity in cultured A431 cells (14). Additionally, cell cycle distribution did not show significant differences in genistein-treated cells, indicating that the phosphorylation status of cell-cycle-controlling enzymes did not become altered at these low genistein concentrations. These data do not support the first postulated mechanism of action.

Since the ER- cells (MDA-MB-468) were as susceptible to differentiation induction as the ER+ cells (MCF-7), it is suggested that genistein initiates this process independently of the ER and possibly independently of its estrogenic/antiestrogenic action. We also found that genistein-treated MDA-MB-468 (ER-) cells were unable to give rise to tumors in nude mice (unpublished data), providing further support to the conclusion that the estrogenic properties of genistein can be dissociated from its differentiation-inducing properties in breast cancer cells. These observations contradict the second mechanism proposed above. Although in the present study we do not provide direct evidence for the occurrence of dynamic changes in chromatin structure following genistein treatment, our earlier studies demonstrated a clear effect of genistein on chromosomal DNA through the enzyme DNA topoisomerase II (3-5). Furthermore, hexamethylene bis-acetamide (HMBA), another inducer of cell differentiation, has been shown to induce dynamic changes in chromatin structure through top II (30). Also, a large number of anti-tumor drugs are known to form a complex with top II and induce differentiation at subcytotoxic concentrations (31). These studies provide support to the third proposed mechanism, in which dynamic changes in chromatin structure play a crucial role in the pathway leading to cell differentiation in genistein-treated tumor cells.

Hundreds of effective inducers of cell differentiation have been reported in the literature, but only a few have found applications in the clinic. This may be due to the high degree of cell selectivity of these agents, which limits their clinical use. For instance, many of these inducers are effective on specific tumor cell lines or variants that are grown only in culture. Another limitation to the clinical applications of differentiation therapy is the narrow range of toxicity which many differentiation inducers demonstrate. Genistein’s ability to induce mature phenotype of normal developing end buds and terminal buds (22) suggests that it can find application in cancer prevention. The wide spectrum of tumor cells that respond to genistein’s maturation-inducing property and its documented toxicity only at doses that cannot be reached physiologically, makes it an excellent candidate for differentiation therapy. These theoretical considerations in conjunction with our studies strongly suggest that genistein, like tamoxifen or retinoids, can find applications in differentiation therapy.

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References


