Soya phytonutrients act on a panel of genes implicated with BRCA1 and BRCA2 oncosuppressors in human breast cell lines

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Breast cancer is the most common cancer in women and a significant cause of death. Mutations of the oncosuppressor genes BRCA1 and BRCA2 are associated with a hereditary risk of breast cancer, and dysregulation of their expression has been observed in sporadic cases. Soya isoflavones have been shown to inhibit breast cancer in studies in vitro, but associations between the consumption of isoflavone-containing foods and breast cancer risk have varied in epidemiological studies. Soya is a unique source of the phytoestrogens daidzein (4',7-dihydroxyisoflavone) and genistein (4',5,7-trihydroxyisoflavone), two molecules that are able to inhibit the proliferation of human breast cancer cells in vitro. The aim of the present study was to determine the effects of genistein (5 μg/ml) and daidzein (20 μg/ml) on transcription in three human breast cell lines (one dystrophic, MCF10a, and two malignant, MCF-7 and MDA-MB-231) after 72 h treatment. The different genes involved in the BRCA1 and BRCA2 pathways (GADD45A, BARD1, JUN, BAX, RB1, ERα, ERβ, BAP1, TNFa, p53, p21, p53, RAD51, Ki-67) were quantified by real-time quantitative RT-PCR, using the TaqMan method and an ABI Prism 7700 Sequence Detector (Applied Biosystems). We observed that, in response to treatment, many of these genes were overexpressed in the breast cancer cell lines (MCF-7 and MDA-MB-231) but not in the dystrophic cell line (MCF10a).

Genistein: Daidzein: BRCA1: BRCA2: Real-time quantitative RT-PCR

Introduction

Breast cancer is a very common disease and is constantly increasing in incidence among women. In spite of increasingly early detection, and better treatment, this cancer remains the principal cause of mortality in women in France.

Cancer, in particular of the breast, has a multifactorial origin. Two major genes, BRCA1 (Miki et al. 1994) and BRCA2 (Wooster et al. 1995), have been implicated in the inherited predisposition to breast and ovarian cancers. Germ-line mutations in BRCA1 and BRCA2 are believed to be responsible for 5–10% of all breast cancer cases (Claus et al. 1991). In sporadic human breast cancers, despite the fact that somatic mutations have not been detected (Futreal et al. 1994) alterations of the levels of BRCA1 and BRCA2 mRNA have been observed (Thompson et al. 1995). Environmental factors are also important. Indeed, the incidence of breast cancer varies according to geographical location, being lower in Asian countries than in North America or Europe. This suggests an important effect of lifestyle, particularly of dietary practices, in the genesis of breast cancer (Greenstein et al. 1996).

Several epidemiological studies assessing the association between soy or isoflavone consumption and breast cancer risk have been reported. So far, there have been epidemiological studies directly assessing the relationship between the dietary intake of soya and the risk of breast cancer. Two case control studies have shown clear protective effects for frequent soya consumption only in premenopausal women (Lee et al. 1991; Hirose et al. 1995). Other studies have not found differential effects for pre- and postmenopausal women (Yuan et al. 1995; Wu et al. 1996; Dai et al. 2001; Horn-Ross et al. 2001; Shu et al. 2001), and most of these studies have not reported a protective effect for frequent soya consumption (Yuan et al. 1995; Wu et al. 1996; Witte et al. 1997; Dai et al. 2001). The case control study of Wu et al. (1996) showed protective effects, but these were significant only in non-US-born Asian-Americans. Grace et al. (2004) recently reported that exposure to soya isoflavones was associated with an increased risk of breast cancer in a UK prospective study.

Soya contains the isoflavones genistein and daidzein in the form of their glycosidic conjugates (Peterson, 1995), and it has been proposed that these isoflavones can prevent breast cancer (Fotis et al. 1993; Lamartiniere et al. 1995). Several experimental studies have reported daily median total isoflavone intakes in Japan of 47.2 mg/d (Arai et al. 2000), in China of 39.26 mg/d (Chen et al. 1999), in US non-Asian (African American, Latin and white) women of 2.872 mg/d (Horn-Ross et al. 2000), and in Europe of less than 2 mg/d (Keinan-Boker et al. 2002). Circulating concentrations of genistein do not, however, generally exceed 10 μM (~3 μg/ml;...
Genistein at a concentration of 10–100 μM (3–30 μg/ml) inhibits the growth and survival of cancer cells derived from the human breast (Zava & Duwe, 1997; Fioravanti et al., 1998; Hsieh et al., 1998; Le Bail et al., 1998). The growth-inhibitory effects of genistein may be linked to its ability to elicit apoptosis (Shao et al., 1998; Li et al., 1999; Balabhadrapathruni et al., 2000). The mechanisms by which genistein modulates the growth and survival of breast cells and prevents breast cancer are not fully understood. As genistein is a phytoestrogen, it may influence cell growth via an inhibitory or stimulatory interaction with the oestrogen receptor (Kuiper et al., 1997, 1998). It also possesses antioxidant properties (Barnes & Peterson, 1995) and angiogenetic (Fotsis et al., 1993) and cell cycle regulatory molecules (Choi et al., 1998; Shao et al., 1998), all of which may be involved in its chemopreventive activity.

Genistein and daidzein inhibited the growth of tumour cell lines and various chemically induced tumours in different animal models (Kim et al. 1998; Lamartiniere et al. 2002; Nomoto et al. 2002). The mechanisms underlying this protective effect are still, however, poorly elucidated. The activity of these molecules may result from their structural homology to 17β-oestradiol, which enables them to bind and activate the oestrogen receptor.

In order to better understand the mechanisms of action of genistein and daidzein, we studied their effects on the expression of the BRCA1 and BRCA2 genes, which are highly involved in breast cancer. BRCA1 protein regulates multiple nuclear processes including DNA repair, cell cycle checkpoints and transcription (Welch et al. 2000). These activities require interaction with other proteins. BRCA1 is associated with BRCA2, a protein implicated in the same nuclear processes (Chen et al. 1998). The activity of these two genes is coordinated by interactions with other genes, including GADD45A, BARD1, JUN, BAX, Rb1, oestrogen receptor (ERα), ERβ, BAP1, TNF, p53, p21/Waf1/Cip1, p300, RAD51, p53, and Ki-67.

Oestrogen receptor α and β modulate the expression of BRCA1 and BRCA2 indirectly (Marks et al. 1997; Fig. 1). BRCA1 also inhibits the transactivation activity of these receptors, when they are activated, by binding to the AF-2 domain of the oestrogen receptor (Fan et al. 2001).

Proteins p53 and p21/Waf1/Cip1 are both involved in cell cycle control, blocking mitosis and inducing apoptosis when DNA is damaged. The expression of p21/Waf1/Cip1 is controlled by p53, as well as by BRCA1 in p53-dependent or p53-independent pathways.

BRCA1 is also able to induce the expression of p53. Jun encodes the c-jun protein, which with fos forms the activated protein-1 transactivator. A functional interaction has been described between BRCA1 and this complex, with a role in the transcriptional regulation of various genes.

Rb1 protein is a transcription factor involved in retinoblastoma and cell cycle control. Rb1 seems to take part in the BRCA1 response pathway, in particular in the cell cycle arrest induced by an increased expression of BRCA1.

p300 takes part in various protein complexes involved in transcriptional regulation. In interacting with CBP protein, p300 modulates the inhibition that BRCA1 exerts on the activity of the oestrogen receptor (Fan et al. 2002). The p300/CBP complex can also bind to BRCA1 and functions as a BRCA1 transcriptional co-factor (Pao et al. 2000).

RAD51 is a homologue of the Escherichia coli RecA gene, which is involved in homologous recombination. RAD51 forms a complex with BRCA1 and BRCA2, allowing the repair of double-stranded DNA breaks by homologous recombination.

GADD45A (growth arrest and DNA damage-inducible gene α) protein is involved in the nucleotide excision repair pathway of DNA repair. This protein interacts with the p53 pathway and permits G2/M transition in the presence of DNA damage. Upregulation of this gene by BRCA1 has been described.

BARD1 (BRCA1-associated ring domain-1) can bind BRCA1 via its BRCT domains. This complex has ubiquitin ligase activity; this may be involved in the repair of DNA damage and proteasome-mediated protein degradation. BAP1 (BRCA1-associated protein-1) exhibits ubiquitin hydrolase activity and forms a complex with BRCA1. BAP1 may potentiate the inhibitory effect of BRCA1 on cancer cell proliferation.

TNFα is a pro-inflammatory cytokine with various activities, such as inhibition of cancer cell proliferation and activation of transcription factor-activated protein-1. The transcription of TNF is induced by BRCA1.

The p52 gene is a marker of the activated oestrogen receptor pathway because the expression of this gene is induced by 17β-oestradiol or oestrogen agonists. This gene was selected to investigate whether the effects of two phytoestrogens were mediated by the oestrogen receptor.

Ki-67 is a cell proliferation marker whose expression is maximal at the end of the G1 phase and in S, G2, and M phases. Ki-67 could not be detected in cells because of its low expression.

BAX (Bcl2-associated X protein) is a pro-apoptotic protein. Its transcription can be activated by p53 during DNA repair. A study has shown that BRCA1 increases the transcription of this gene induced by p53 (Zhang et al. 1998).

Fig. 1 shows the biological pathways in which all these genes are involved. We quantified the expression of these
genes by quantitative RT-PCR in three human cell lines (MCF10a, MDA-MB-231, MCF-7) untreated or treated for 72 h with 5 μg/ml genistein or 20 μg/ml daidzein.

Materials and methods

Cell culture

The breast cancer cell lines MCF-7 (Soule et al. 1973) and MDA-MB-231 (Calléau et al. 1974), and the fibrocyctic mammary cell line MCF10a (Soule et al. 1990), were obtained from the American Type Culture Collection. Oestrogen receptor α and β status was determined by immunohistochemistry by the Centre Jean Perrin’s anatomopathologist. MCF-7 was found to be ERα+/ERβ−, MDA-MB-231 was ERα+/ERβ−, and MCF10a was ERα−/ERβ− (Le Corre et al., 2004).

MCF-7 cells were cultured in RPMI 1640 medium supplemented with 2 mM-glutamine, 1% gentamicin and 10 μg/ml insulin (Sigma) in a humidified atmosphere at 37°C containing 5% CO2. MCF10a cells were maintained in DMEM/Ham F-12 medium containing 10% horse serum (Life Technologies), 2 mM-glutamine, 1% gentamicin, 10 μg/ml insulin, 20 ng/ml epidermal growth factor (Sigma), 100 ng/ml cholaer toxin (Sigma) and 0.5 μg/ml hydrocortisone (Sigma) at 37°C with 5% CO2. MDA-MB-231 cells were grown in Leibovitz 15 medium, with 2 mM-glutamine, 1% gentamicin and 15% fetal bovine serum, in a 37°C humidified atmosphere without CO2. In our laboratory, we previously tried to use charcoal stripped for cell culture and, at the same time, medium without phenol red to avoid the presence of oestrogen. The results were similar (data not shown).

Cell treatments

Each cell line was plated at 1 × 10⁶ cells per T-75 flask. Cell proliferation assay had previously been carried out for each phytoestrogen, producing the dose–response curve. The concentration leading to a 50% inhibition of proliferation was determined: 5 μg/ml genistein (4,5,7-trihydroxyisoflavone, Sigma) dissolved in dimethylsulphoxide or 20 μg/ml daidzein (4,7-dihydroxyisoflavone, Sigma) dissolved in dimethylsulphoxide. Cells were treated for 72 h (Bernard-Gallon et al., 1998; Vissac-Sabatier et al. 2003).

RNA isolation

After 72 h treatment, cells at 70–80% confluence in the T-75 flasks were extracted, and total RNA was isolated using 5 ml RNA-Plus reagent (QBiogene) after three washes with PBS, according to the manufacturer’s protocol. The quality of RNA was verified by electrophoresis on a Bioanalyzer 2100 (Agilent).

Reverse transcription

RNA was reverse-transcribed in a final volume of 16 μl containing 0.2 μg/μl random hexamers, 200 mM-dithiothreitol, 1X bulk First Strand Mix (Amersham) and 5 μg RNA. The RNA and diethylpyro-carbonate water were first incubated at 65°C for 10 min; then the random primer, bulk buffer and dithiothreitol were added, and the samples were incubated at 37°C for 1 h. Reverse transcriptase was inactivated by heating at 95°C for 5 min.

Real-time quantitative RT-PCR

Multiplex PCR was carried out on cDNA in ninety-six-well plates. To the 25 ng cDNA were added 20 μl of reaction mix containing 12.5 μl TaqMan universal PCR Master Mix (Roche); containing dATP, dCTP, dGTP and dUTP, MgCl2, AmpliTaqGold, amperase uracil-N-glycosylase, 200 μM-TaqMan probes corresponding to the studied genes, 400 nM of each primer and 50nM 18S rRNA primers and TaqMan probe.

For all gene quantitative expressions, probes and primers were designed so that they overlapped splice junctions, thereby avoiding the potential amplification of genomic DNA. The sequence of forward primers, TaqMan probes and reverse primers for BRCA1, BRCA1 exon 11, BRCA2, BRCA2 exon 12, ERα, p53, p21Waf1/Cip1, p300, RAD51, p52 and Ki-67 genes are described in Table 1. To assay the expression of Rhl (Hs00153126_m1), GADD45A (Hs00169255_m1), TNFa (Hs00174128_m1), BAX (Hs00180269_m1), BARD1 (Hs00184427_m1), BAP1 (Hs00184962_m1), ERβ (Hs00230957_m1) andJun (Hs00277190_m1) genes, Assays-on-Demand (Applied Biosystems) were used. All doubly labelled probes, 18S rRNA probe and primers, and TaqMan universal PCR Master Mix were obtained from Applied Biosystems.

Thermal cycling conditions were 2 min at 50°C and 10 min at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Data were collected using an ABI Prism 7700 SDS analytical thermal cycler (Applied Biosystems).

Relative gene expression was determined using the comparative threshold cycle method, which consists of normalising the number of target gene copies to an endogenous reference gene (18S rRNA), designated as the calibrator (Fink et al. 1998). The level of each mRNA in each treated cell line was then normalised to the result obtained in the untreated cells. The quantification of gene expression using real-time quantitative RT-PCR can be performed using the comparative threshold cycle method or the standard curve. We have previously compared these two strategies for BRCA1 transcripts in human cancer breast cell lines, finding no difference (Fay et al. 2000).

To guarantee reproducibility, two independent extractions of total RNA were done. Two independent reverse transcription were carried out for one RNA extraction, whereas only one was performed for the second extraction. Each reverse transcription was analysed in triplicate and expressed as a mean and standard deviation (Fay et al. 2000).

mRNA for nineteen genes was quantified, corresponding to two splice variants of BRCA1 and BRCA2 (BRCA1 exon 11, BRCA2 exon 12), BRCA1 full length, BRCA2 full length, ERα, ERβ, p52, p53, p21Waf1/Cip1, GADD45, BAX, jun, Rhl, p300, BARD1, BAP1, Rad51, TNFa and Ki-67. This was carried out in three human cell lines (MCF10a, MDA-MB-231, MCF-7) to observe variations of the expression of these genes in response to genistein or daidzein after 72 h incubation. The change in mRNA expression was evaluated by real-time TaqMan quantitative RT-PCR using the ΔΔCt.
Phytoestrogens and breast cell gene expression

Table 1. Description of oligonucleotide primer pairs and TaqMan probes

<table>
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<th>Genes</th>
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<tr>
<td>BRCA1 exon 11</td>
<td>5'-1790GCCCCAACCAAAAGAAAGTCT-3'</td>
<td>5'-309AAGAGGAACGGGCTTGGAA-3'</td>
<td>5'-519AAGAGGAACGGGCTTGGAA-3'</td>
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<td>BRCA1 exon 12</td>
<td>5'-1810CATCAAGGAACAGCCTCAACCATCAGGA-3'</td>
<td>5'-5417AAATTAATAAGCAAGAAGCAAAGCATGGATTCAAACTTA-3'</td>
<td>5'-2143AAATTAATAAGCAAGAAGCAAAGCATGGATTCAAACTTA-3'</td>
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<td>BRCA2 isoform of the oestrogen receptor</td>
<td>5'-409AAGAGGAACGGGCTTGGAA-3'</td>
<td>5'-5417AAATTAATAAGCAAGAAGCAAAGCATGGATTCAAACTTA-3'</td>
<td>5'-2143AAATTAATAAGCAAGAAGCAAAGCATGGATTCAAACTTA-3'</td>
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<td>5'-2143AAATTAATAAGCAAGAAGCAAAGCATGGATTCAAACTTA-3'</td>
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<td>Ki-67</td>
<td>5'-409AAGAGGAACGGGCTTGGAA-3'</td>
<td>5'-5417AAATTAATAAGCAAGAAGCAAAGCATGGATTCAAACTTA-3'</td>
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<td>ERa</td>
<td>5'-409AAGAGGAACGGGCTTGGAA-3'</td>
<td>5'-5417AAATTAATAAGCAAGAAGCAAAGCATGGATTCAAACTTA-3'</td>
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<td>ERa</td>
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Primer and probe sequences (5'-3') used for mRNA quantification by real-time quantitative RT-PCR. Sequences were designed with Primer Express software (Perkin-Elmer Applied Biosystems). aNumbers indicate the nucleotide position of oligonucleotides in the gene cDNA sequence (GenBank accession numbers: BRCA1, U14680; BRCA2, U14681; p53, U94788; p21 WAF1, X00474; Ki-67, X65550).

Statistical analysis

Each value represents the mean and standard deviation for three assays. Statistical analysis of the results was carried out by Student’s t-test, differences being considered significant at P<0.05.

Results

MCF 10a, which does not exhibit ERB, expressed mRNA encoding ERα (Fig. 2). In response to genistein, the level of BRCA1, TNF (P<0.001), BARD1, p53, pS2, jun, BAX, BAP1 and BARD1 (P<0.05) mRNAs were significantly increased in comparison with control values. BRCA2 mRNA was significantly decreased. Conversely, p53, pS2, jun, GADD45 (P<0.001), BAX, TNF, Rb1 and p300 (P<0.05) mRNAs were significantly decreased in comparison with the control in the presence of daidzein.

MDA-MB-231 cells, known to exhibit the β and not the α isoform of the oestrogen receptor, only exhibited mRNA for ERβ. Genistein 5 μg/ml significantly increased mRNA expression for the majority of the genes studied in comparison with cell control. Only pS2 mRNA decreased significantly (P<0.001) in the presence of genistein when compared with the cell control. BRCA1 exon 11, BRCA2, BRCA2 exon 12 and ERβ exhibited no change in comparison with the cell control (Fig. 2).

Daidzein 20 μg/ml increased the expression of the majority of these genes. ERβ and BRCA2 exon 12 mRNA decreased significantly in comparison with the control (P<0.001 and P<0.05, respectively), whereas there was no variation for BRCA1 exon 11, pS2, jun or TNF. It should be noted that p21Waf1/Cip1, BARD1, GADD45α (P<0.001), RAD51 and p300 (P<0.05) were significantly increased in comparison with the cell control.

MCF-7 cells exhibited both the ERα and ERβ isoforms, which was confirmed by our assay of ERα and ERβ mRNA (Fig. 2). In these cells, genistein significantly increased pS2 (P<0.05), p21Waf1/Cip1, BAX and GADD45 (P<0.001) mRNA. Daidzein 20 μg/ml significantly increased all the genes studied in comparison with control.

Discussion

Our purpose in the present study was to determine the effects of genistein and daidzein on the expression of a panel of genes coding for proteins interacting with the functions of BRCA1 and BRCA2, in order to better understand the mechanisms by which these two soya-derived phytoestrogens may protect against breast cancer. So far, only epidemiological studies have been carried out; nothing is known about the mechanism involved.

Previously, a dose–response curve was established for the phytoestrogens genistein (2.5, 5, 10, 20, 30, 40 μg/ml) and
daidzein (10, 20, 30, 40, 50, 60 μg/ml). The concentration leading to a 50% inhibition in proliferation was selected for further treatment: 5 μg/ml for genistein and 20 μg/ml for daidzein (Vissac-Sabatier et al. 2003). In most studies, the concentration of genistein used has been more than 25 μM (Takahashi et al. 2004). Circulating concentrations of genistein do not, however, generally exceed 10 μM (Coward et al. 1996; King & Bursill, 1998; Izumi et al. 2000; Hong et al. 2002). In this respect, molecular data are available on the effects of genistein on breast cancer cells at concentrations less than 25 μM (Takahashi et al. 2004).

We analysed mRNA for several genes using real-time quantitative RT-PCR. Three human breast cell lines with different oestrogen receptor status were used: MCF-7, a breast tumour cell line (ERα+/ERβ+); the breast tumour cell line MDA-MB-231 (ERα2/ERβ+); and the fibrocystic breast cell line MCF10a (ERα2/ERβ2). For MCF10a (Fig. 2), very low variations (0.5–1.5) in gene expression were obtained. Only BARD1 mRNA was strongly increased in response to genistein. These results are in agreement with those of our previous studies, which showed a reduced sensitivity of MCF10a cells to other micronutrients such as fatty acids (Bernard-Gallon et al. 2002) and resveratrol (Le Corre et al. 2004). The non-malignant status of MCF10a, as well as the absence of ERα and ERβ, might explain why these cells are less sensitive to the action of genistein and daidzein.

Genistein has several properties: agonist/antagonist oestrogenic activity; the capacity to inhibit phosphotyrosine kinase and topoisomerase II; cytotoxic and mutagenic activities in diverse cell lines. The effects of genistein on the cell lines MCF-7 and MDA-MB-231 might be the result of these activities.

In the MCF-7 cells, genistein seems to act as an oestrogen agonist, as demonstrated by the increase in pS2 mRNA, which resulted in a decrease in ERα mRNA, probably owing to negative feedback from ERα receptors. This mechanism has been reported in a previous study (Maggiolini et al. 2001).

This oestrogenic effect does not, however, induce the expression of BRCA1 and BRCA2. Indeed, the induction of these genes in the presence of oestrogen agonists is indirect and is probably the result of mitogenic effects and the blocking of cells in G2/M phase (Marks et al. 1997; Nomoto et al. 2002). Moreover, 5 μg/ml genistein does not seem to induce this cell cycle arrest, which may explain the absence of

![Fig. 2](https://www.cambridge.org/core/core/terms, https://doi.org/10.1079/BJN20051640)
induction of BRCA1 and BRCA2. This is confirmed by the unchanged expression of Ki-67, indicating that the cells have normal growth.

In contrast, in MCF-7, 5 μg/ml genistein increased p21Waf1/Cip1 and BAX expression. This activation seems to be independent of p53, whose expression did not vary. The induction of BAX and p21Waf1/Cip1 by genistein, independently of p53, has already been reported in other cell lines (Alhasan et al. 2001).

GADD45 mRNA was also induced by genistein in MCF-7 cells. Like BAX and p21Waf1/Cip1, GADD45 takes part in cell cycle control and the induction of apoptosis after DNA damage. The effect of genistein on the expression of these genes might be explained by its mutagenic and cytotoxic properties (Morris et al. 1998; Maggiolini et al. 2001). Indeed, at concentrations higher than 1 μg/ml, genistein causes nuclear damage, whereas at lower concentrations, its effects are exclusively oestrogenic. The cytotoxic activity of genistein also seems to depend on the ERβ receptor, which is expressed in MCF-7 cells (Maggiolini et al. 2001).

A study concerning the effects of an anti-cancer molecule (NB1011) corroborates the hypothesis of the cytotoxicity of genistein. Neuteboom et al. (2002) demonstrated that the cytotoxic activity of NB1011 induced the expression of BAX, p21Waf1/Cip1, and GADD45 mRNA, similar to the effect of genistein in MCF-7 cells.

The effect of genistein on MCF-7 seems to be the result of oestrogenic effects via ERα, as well as cytotoxic effects dependent on ERβ. The results of these two different effects lead to the hypothesis of a possible counterbalance, which would explain why the majority of genes do not vary in their expression. The oestrogenic activity induced proliferation, whereas the cytotoxicity inhibited growth. Thus, MCF-7 cells did not modify their cell cycle, or block their growth, which is confirmed by the lack of variation in Ki-67 mRNA.

MDA-MB-231 cells were more responsive than MCF-7 to genistein, with an increased expression of most of the genes studied. This difference may be due to the different oestrogen receptor status of these two cell lines. MDA-MB-231 cells (ERα/ERβ) exhibited ERβ mRNA, and ERβ protein has been clearly identified elsewhere; it may thus be supposed that genistein acts independently or as an antagonist of the oestrogen pathway, as suggested by the fall in p53 mRNA. On the other hand, the cytotoxic effects of genistein are exhibited by an increased expression of all the genes involved in cell cycle control, apoptosis and DNA repair, such as BRCA1, p53, p21Waf1/Cip1, Rad51, BARD1, BAX, GADD45 and Rbl1. This hypothesis is supported by the fact that MDA-MB-231 cells do not show ERα, but exhibit ERβ which is a mediator of the cytotoxic effects of genistein. Moreover, 5 μg/ml genistein blocked most MDA-MB-231 cells in G2/M, probably owing to an overexpression of GADD45 (Shao et al. 1998; Jin et al. 2002) following the cellular and nuclear damage caused by genistein. This arrest may explain the increase in Ki-67 mRNA, which is overexpressed in G2/M.

The antagonist oestrogenic action of genistein may explain the increase of jun and TNF mRNA in MDA-MB-231. Oestrogens are known to inhibit the expression of the TNF and jun genes via the ERβ (Srivastava et al. 1999). Genistein seems to decrease the inhibition exerted by oestrogens on the transcriptions of these genes by antagonising ERβ.

The only difference between genistein and daidzein is the presence of a hydroxyl group on carbon 5 of genistein, this structural difference influencing the properties of these isoflavones. Whereas genistein has several biological activities, including an inhibition of phosphotyrosine kinase and topoisomerase II, oestrogenic activity and cytotoxicity, daidzein is known only to bind and activate the oestrogen receptor.

Sathyamoorthy & Wang (1997) demonstrated that daidzein was able to inhibit the growth of MCF-7 cells by blocking their cell cycle in G1 phase. This would explain the increase we observed in Ki-67 mRNA, as Ki-67 is overexpressed in G1 phase.

In MCF-7, daidzein seems to bind and activate the oestrogen receptor, as corroborated by the increase in p52 mRNA. This activation of the oestrogen pathway could be the basis of the overexpression of BRCA1 and BRCA2 (Spillman & Bowcock, 1996; Marks et al. 1997). In MDA-MB-231, no induction of the p52 gene was found, suggesting that the oestrogen pathway was not activated. The increase in BRCA1 and BRCA2 RNA must be caused by another pathway, which remains to be determined. This also demonstrates that daidzein is an oestrogen agonist, mainly in cells exhibiting ERα.

Daidzein also increased the level of p53 mRNA in MCF-7 and MDA-MB-231. This result may be explained by the overexpression of BRCA1, which can indirectly activate the transcription of p53. p53 protein is a major factor in the control of the cell cycle and induces the expression of different genes involved in cell cycle arrest and apoptosis, such as p21Waf1/Cip1 and BAX. p21Waf1/Cip1 and BAX were also induced by daidzein, probably in response to the activation of p53. GADD45 and Rbl1 are also genes under the transcriptional control of BRCA1 (Aprelikova et al. 1999). It seems obvious to see them overexpressed in the presence of daidzein conjointly with BRCA1 induction.

Several points are raised by this study. First, an extensive analysis of the effects of genistein and daidzein on a larger panel of genes interacting with BRCA1 and BRCA2 might be performed by transcriptome biochip technology or by the use of microarray membranes. Second, our results provide a basis for further elucidation of the cancer-preventing effect of high concentrations of isoflavones. In addition, differences in the results from three cell lines with a different oestrogen receptor status and using a panel of genes implicated in BRCA1 and BRCA2 oncosuppression suggest that we have yet to fully delineate the biological effects of these phytochemicals.

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References


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Wiseman RW, Futreal PA & Iglehart JD (1997) BRCA1 is a more bioavailable soymilk isoflavone than is genistein in adult women. J Nutr 127, 825–832.

