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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*, *TNFα*, *p53*, *p21* $^{Waf1/Cip1}$, *p300*, *RAD51*, *pS2*, *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. Germline 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 (Fotsis *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;

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Coward *et al.* 1996; King & Bursill, 1998; Izumi *et al.* 2000; Hong *et al.* 2002), with a corresponding value of between 0.3 and 3.5 μM for Caucasian populations (Xu *et al.* 1994).

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 (Welcsh *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* $^{Waf1/Cip1}$*i*, *p300*, *RAD51*, *pS2* and *Ki-67*.

Oestrogen receptor α and β modulate the expression of *BRCA1* and *BRCA2* indirectly (Marks *et al.* 1997; Fig. 1).

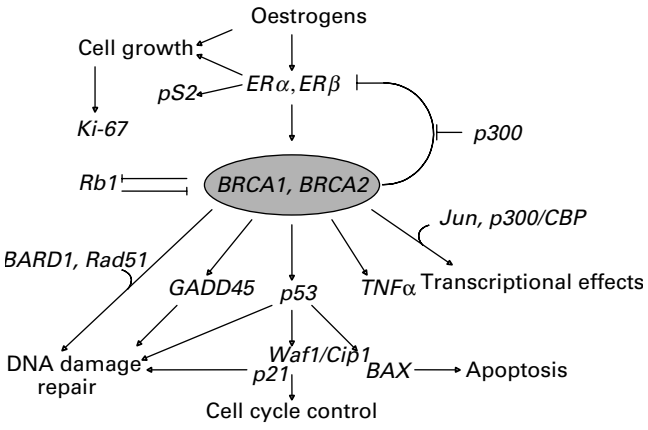

Fig. 1. *BRCA1*, *BRCA2* and the other genes studied: interactions and implications for cell processes.

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 G_2/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 *pS2* 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 G_1 phase and in S, G_2 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 (Cailleau et al. 1974), and the fibrocystic 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 $ER\alpha^{+}/ER\beta^{+}$, MDA-MB-231 was $ER\alpha^{-}/ER\beta^{+}$, and MCF10a was $ER\alpha^{-}/ER\beta^{-}$ (Le Corre *et al.*, 2004).

MCF-7 cells were cultured in RPMI 1640 medium supplemented with 2 mM-glutamine (Sigma), 1 % gentamicin (Sigma), 10 % fetal bovine serum (Life Technologies) and 10 μg/ml insulin (Sigma) in a humidified atmosphere at 37°C containing 5 % CO_2. 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 cholera toxin (Sigma) and 0.5 μg/ml hydrocortisone (Sigma) at 37°C with 5 % CO_2. 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 CO_2. 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 1×10^6 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, $MgCl_2$, AmpliTaqGold, amperase uracil-N-glycosylase, 200 nM-TaqMan probes corresponding to the studied genes, 400 nM of each primer and 50 nM 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*, *p21 Waf1/Cip1*, *p300*, *RAD51*, *pS2* and *Ki-67* genes are described in Table 1. To assay the expression of *Rb1* (Hs00153126_m1), *GADD45A* (Hs00169255_m1), *TNFα* (Hs00174128_m1), *BAX* (Hs00180269_m1), *BARD1* (Hs00184427_m1), *BAP1* (Hs00184962_m1), *ERβ* (Hs00230957_m1) and *jun* (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 (Favy *et al.* 2000).

To guarantee reproducibility, two independent extractions of total RNA were done. Two independent reverse transcriptions 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 (Favy *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*, *pS2*, *p53*, *p21 Waf1/Cip1*, *GADD45*, *BAX*, *jun*, *Rb1*, *p300*, *BARD1*, *BAP1*, *Rad51*, *TNFα* 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

Table 1. Description of oligonucleotide primer pairs and TaqMan probes

Genes	Forward primers	TaqMan probe	Reverse primers
BRCA1	^{5566a}CAGAGGACAATGGCTTCCATG^{5586}	^{5588}AATTGGGCAGATGTGTGAGGCACCTG^{5613}	^{5646}CTACACTGTCCAACACCCACTCTC^{5623}
BRCA1 exon 11	^{4157}AAGAGGAACGGGCTTGGAA^{4175}	^{4177}AAAATAATCAAGAAGAGCAAAGCATGGATTCAAACTTA^{4214}	^{4236}CACACCCAGATGCTGCTTCA^{4217}
BRCA2	^{9794}CCAAGTGGTCCACCCCAAC^{9812}	^{9818}ACTGTACTTCAGGGCCGTACACTGCTCAAA^{9847}	^{9895}CACAATTAGGAGAAGACATCAGAAGC^{9870}
BRCA2 exon 12	^{7120}GAAAATCAAGAAAAATCCTTAAAGGCT^{7147}	^{7153}AGCACTCCAGATGGCACAATAAAAGATCGAAG^{7184}	^{220}GTAATCGGCTCTAAAGAAACATGATG^{719}
p53	^{1191}CCCAGCCAAAGAAGAAACCA^{1210}	^{1227}TCACCCTTCAGATCCGTGGGCG^{1248}	^{1292}GTTCCAAGGCCTCATTCAGCT^{1272}
p300	^{5213}ACCGTGGAAGTAAAACCAGGC^{5233}	^{5234}TGAAAGCAAGGTTTGTGGACAGTGGAGA^{5262}	^{5286}CGGTATGGAAAGGATTCTGCC^{5265}
Rad51	^{659}GAGGTGAAGGAAAGGCCATGT^{679}	^{725}TGGCTGAGAGGTATGGTCTCTCTGGCA^{751}	^{808}GGGTCTGGTGGTCTGTGTTGA^{788}
p21$^{Waf1/Cip1}$	^{183}TGAGCCGCGACTGTGATG^{190}	^{204}TAATGGCGGGCTGCATCCAGGA^{215}	^{264}GTCTCGGTGACAAAGTCGAAGTT^{242}
pS2	^{205}CCTCCCAGTGTTCGACGACACCGTTCG^{228}	^{2089}CGCATTCCTTGCAAAAGTATTACATCACGG^{2118}	^{274}TGGAGGGACGTCGATGGTAT^{255}
ERα	^{2068}CCACTGCGGGCTCTACTTCA^{2087}	^{1846}CATCAAGGAACAGCCTCAACCATCAGGA^{1873}	^{2143}GTGGCAGGGAAACCCTCTG^{2125}
Ki-67	^{1790}GCCCCAACCAAAAGAAAGTCT^{1810}	TGCTGGCACCAGACTTGCCCTC	^{1922}AGCTTTGTGCCTTCACTTCCA^{1902}
18S	CGGCTACCACATCCAAGGAA		GCTGGAATTACCGCGGCT

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). [a]Numbers indicate the nucleotide position of oligonucleotides in the gene cDNA sequence (GenBank accession numbers *BRCA1*, U14680; *BRCA2*, U43746; *p53*, U94788; *p21* $^{WAF1/CIP1}$, BC000312; *ERα*, X03635; *ERβ*, AF051427; *RAD51*, D14134; *pS2*, X00474; *Ki-67*, X65550).

method and normalisation to the values in the untreated cells. Each value represents the mean and standard deviation for three assays. For each condition, two independent mRNA extractions were carried out, followed by three reverse transcriptions (two independent reverse transcriptions for one extraction, one reverse transcription for second extraction).

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 ERβ, 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 *p21* $^{Waf1/Cip1}$, *BARD1*, *GADD45a* ($P<0.001$), *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* ($P<0.05$), *p21* $^{Waf1/Cip1}$, *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

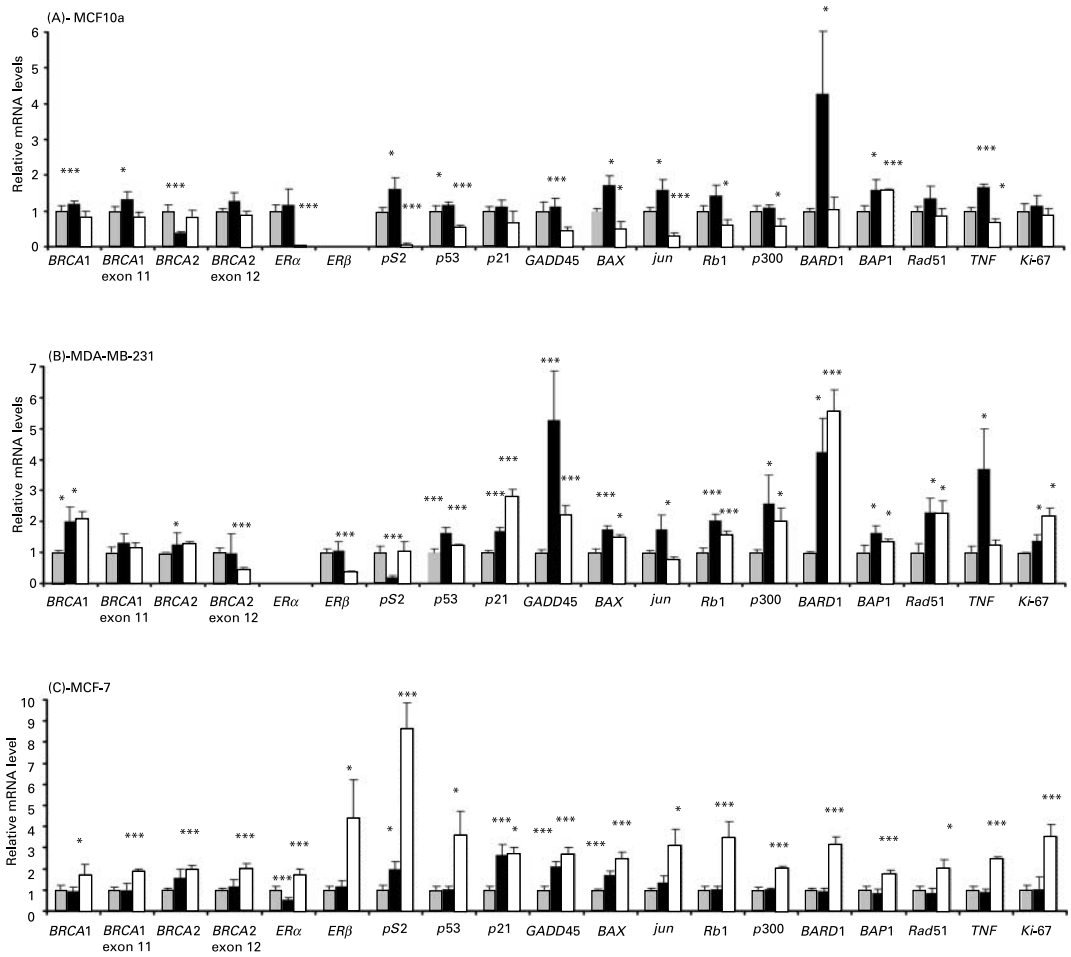

Fig. 2. The relative expressions of *BRCA1*, *BRCA1* exon 11, *BRCA2*, *BRCA2* exon 12, *ERα*, *ERβ*, *pS2*, *p53*, *p21*, *GADD45A*, BAX, *jun*, *Rb1*, *p300*, *BARD1*, *BAP1*, *Rad51*, *TNF* and *Ki-67* mRNA were quantified by real-time quantitative RT-PCR in (a) MCF10a, (b) MDA-MB-231 and (c) MCF-7 cells after treatment with genistein (5 μg/ml) or daidzein (20 μg/ml) for 72 h (▩, control; ■, genistein (5 μg/ml); □, daidzein (20 μg/ml)). Target gene expression values are expressed as fold changes compared with untreated cells (defined as 1). Data represent the average of three replicates with their standard errors. $^{*}P<0.05$, $^{***}P<0.001$.

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 (~7 μg/ml; Takahashi *et al.* 2004). Circulating concentrations of genistein do not, however, generally exceed 10 μM (~3 μg/ml; 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 (~7 μg/ml).

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 ($ER\alpha^{+}/ER\beta^{+}$); the breast tumour cell line MDA-MB-231 ($ER\alpha^{-}/ER\beta^{+}$); and the fibrocystic breast cell line MCF10a ($ER\alpha^{-}/ER\beta^{-}$). 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 G_2/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

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 *p21 Waf1/Cip1* and *BAX* expression. This activation seems to be independent of p53, whose expression did not vary. The induction of *BAX* and *p21 Waf1/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 *p21 Waf1/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*, *p21 Waf1/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 ($ER\alpha^-/ER\beta^+$) 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 *pS2* 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*, *p21 Waf1/Cip1*, *Rad51*, *BARD1*, *BAX*, *GADD45* and *Rb1*. 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 G_2/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 G_2/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 transcription 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 G_1 phase. This would explain the increase we observed in *Ki-67* mRNA, as Ki-67 is overexpressed in G_1 phase.

In MCF-7, daidzein seems to bind and activate the oestrogen receptor, as corroborated by the increase in *pS2* 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 *pS2* 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 *p21 Waf1/Cip1* and *BAX*. *p21 Waf1/Cip1* and *BAX* were also induced by daidzein, probably in response to the activation of p53. *GADD45* and *Rb1* 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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