

Genistein-induced apoptosis in MCF-7 cells involves changes in Bak and Bcl-x without evidence of anti-oestrogenic effects

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South-east Asian women have a lower rate of breast cancer compared with their counterparts in western countries and the difference in soyabean consumption has been claimed to be a major contributing factor. Genistein is the most studied phytochemical in the soyabean. An anti-oestrogenic effect is believed to play a crucial part in its chemopreventive mechanism. In the present study, we expressed oestrogen receptor (OR) in an OR-negative cell line, HepG2, to investigate the pro- and anti-oestrogenic effect of genistein on the OR transcriptional activity. Genistein by itself had an estimated concentration that induced 50% of the maximum response (EC₅₀) of 2.5 µM for the binding to OR-α. In these experiments, genistein concentration as high as 50 µM could not reduce the oestrogen response element-driven luciferase activities initiated by oestradiol. Instead, genistein potentiated the OR transactivational activity while cell death was detected. On the other hand, an increased Bak and a reduced Bcl-x(L) was observed at 50 µM-genistein by Western analysis. The combined effect of these two proteins could be important in the apoptotic process. Since plasma genistein >50 µM has never been documented following consuming of soyabean or soyabean products, the present study does not support the notion that dietary soyabean exerts its chemopreventive effect through antagonizing OR.

Genistein: Oestrogen receptor: Anti-oestrogen: Cell death

Breast cancer is one of the most common cancers in women. Women in Asian countries have a lower incidence of breast cancer than those in the West, and the descendants of Asian people who migrated to the USA have lost this favourable characteristic (Ziegler *et al.* 1993). These observations suggest that environment does play a role in the aetiology of breast cancer. Soyabean consumption has been proposed to be a contributing factor, although this protective effect is not consistent in epidemiological studies (Lamartiniere, 2000). The soyabean isoflavone genistein has been widely studied for its chemopreventive effect in different animal models. Rats treated with genistein in their neonatal period have reduced incidence of dimethylbenz[α]anthracene-induced breast cancer (Lamartiniere *et al.* 1995*a,b*). In cell culture studies, genistein in pharmacological doses has been found to inhibit growth of many cell types including mammary cancer cells (Kuo, 1997).

Exposure to oestrogen has been considered a risk factor for breast cancer (Nenci *et al.* 1988), because it promotes breast-cell proliferation. One of the proposed mechanisms is in the redirection of apoptotic pathways. 17β-Oestradiol can reduce the pro-apoptotic Bak level in MCF-7 cells (Leung *et al.* 1998) and enhances cell survival by increasing Bcl-2 (Wang & Phang, 1995). The latter is likely to be due to the direct effect of oestrogen receptor (OR) transactivation, because two functional oestrogen response elements (ORE) have been identified at the *bcl-2* gene promoter region (Perillo *et al.* 2000).

OR is an intranuclear binding protein that has a DNA-binding domain and a ligand-binding domain (Pike *et al.* 2000). When the ligand-binding domain has a ligand bound to it, the OR protein will experience a conformational change and initiate transcription by attaching to an ORE in the gene-specific promoter region. Recently, two subtypes of OR known as α and β have been identified,

Abbreviations: EC₅₀, concentration that produced 50% of the maximum response; FBS, fetal bovine serum; IC₅₀, concentration that caused 50% of inhibition; OR, oestrogen receptor; ORE, oestrogen response element; PS, presenilin.

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and their differences in tissue distribution and ligand affinity have been explored in the treatment of oestrogen-related diseases (Dechering *et al.* 2000).

Since most breast-cancer cells are intuitively expressing either OR- α or OR- β , or both (Suo *et al.* 2001; Tong *et al.* 2002), we selected a hepatic cancer cell line HepG2 to examine the specific effect of genistein on OR- α . This cell line has been shown to be OR-negative and found to be useful in the evaluation of ligand-receptor interaction by expressing a specific OR isoform in the cells (Barkhem *et al.* 1997). In the present study, we investigated the correlation of the anti-oestrogenic effect of genistein *v.* apoptosis. Because of its non-discriminatory cytotoxicity to both OR-negative and -positive cell lines (Shao *et al.* 1998), we hypothesized that an anti-oestrogenic effect was not a determining factor in the apoptosis induced by genistein.

Methods

Chemicals

Genistein was purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were purchased from Sigma if not stated otherwise.

Cell culture

We obtained the hepatocarcinoma cell line HepG2 from American Type Cell Collection (Rockville, MD, USA). The cells were incubated in Phenol Red-free RPMI 1640 media with (/litre): L-glutamine (Gibco BRL, Rockville, MD, USA) 2 μ mol, penicillin 100 000 U, streptomycin 100 mg, fetal bovine serum (FBS; Gibco BRL) 50 ml. The incubator was maintained at 37°C in CO₂-air (5:95, v/v). One week before the beginning of experiments, the cells were switched to the same media with charcoal dextran-treated FBS (50 ml/l; Hyclone, Logan, UT, USA) instead of FBS. The cells were trypsinized and seeded at a density of 10⁵ cells per well in twenty-four-well Costar culture plates (Corning Inc, Corning, NY, USA) 1 d before treatment started.

Apoptotic death assay

A cell death detection ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany) was used for this determination. Cells with a density of 5 \times 10⁴ per well were plated in twenty-four-well plates (four wells per treatment). After treatment for 48 h, the cells were washed once with PBS and lysed with 0.5 ml lysis buffer included in the kit. DNA fragments in the cell lysates were then quantified by ELISA. An identically treated cell culture plate was used for the cell viability determination by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Hansen *et al.* 1989). The apoptotic index was then obtained by normalizing the ELISA value with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-absorbance reading.

Oestrogen receptor- α competitive binding assay *v.* transient transfection reporter assay

Genistein is a competitor with oestrogen for OR-binding in a radiolabelled OR competition assay system (Kuiper *et al.* 1997) and exhibits biphasic effects on cell proliferations (Wang & Kurzer, 1997; Breinholt & Larsen, 1998; Le Bail *et al.* 1998; Shao *et al.* 1998). Since the DNA-binding and ligand-binding domains are separate domains, we investigated the anti-oestrogenic effect of genistein by using a transient transfection cell culture model. This system included a sequential events occurred after the activation of OR.

An oestrogen-receptor(α) competition screening kit was obtained from Wako Chemicals (Osaka, Japan) to quantify the competition between genistein and oestradiol for OR- α binding. Genistein was incubated with 10⁻⁹ M-fluorescein-labelled-oestradiol in a ninety-six-well plate coated with recombinant OR- α for 2 h. After flushing the wells with wash buffer, the fluorescence was read at excitation wavelength 480 nm and emission 530 nm by a fluorescence plate reader (Fluostar Galaxy; BMG Labtechnologies GmbH, Offenburg, Germany).

The transfection procedures were performed as previously described (Casanova *et al.* 1999) with slight modification. Human OR- α expression plasmid and ORE-luciferase reporter plasmid were gifts from Dr Donald McDonnell (Duke University, Durham, NC, USA). HepG2 cells were cultured in twenty-four-well plates and were co-transfected with three different plasmids: ORE-luciferase reporter, OR- α expression and Renilla luciferase control plasmids (Promega Corp., Madison, WI, USA). For each well of cell culture, 0.4 μ g OR- α , 0.4 μ g ORE-luciferase and 0.1 μ g Renilla luciferase control plasmids were mixed with 8 μ l lipofectamine (Gibco BRL) at room temperature for 15 min to form DNA-lipid complexes. The complexes were then mixed in 400 μ l serum-free RPMI and administered to the cultures for 6 h. When the transfection period was over, the complexes-containing medium were replaced by fresh RPMI with charcoal-dextran treated serum (50 ml/l). The cells were treated with genistein and 17 β -oestradiol. After a 24 h treatment, the cells were lysed and assayed for the luciferase activities. The cell lysis buffer and luciferase substrates were provided by a dual-luciferase reporter assay system (Promega). A luminometer (Fluostar Galaxy; BMG Labtechnologies GmbH) was used for dual-luciferase assay. The reporter firefly luciferase activity was measured by adding luciferase assay reagent II from the kit. The firefly luciferase was then quenched by Stop & Glo[®] buffer provided in the kit, followed by the assay of the control Renilla luciferase activity. The firefly luciferase activity was a signal representing OR transactivation activity and the Renilla luciferase reading was employed to normalize the transfection efficiency.

Semi-quantitative reverse transcription-polymerase chain reaction assay

Total RNA was isolated from cells grown in six-well Costar plates (Corning Inc) in triplicate by a method previously

described (Wang & Phang, 1995). RNA (1 μg) was used for cDNA synthesis and the final volume was diluted to 100 μl . A reverse transcription-polymerase chain reaction assay was used to quantify presenilin (PS) 2 mRNA level. Primers of PS2, Bcl-2 and β -actin, sequences already published (Wang & Phang, 1995; Wang *et al.* 1996), were utilized to amplify the target cDNA separately after the first strand reaction. All polymerase chain reaction reactions consisted of 0.2 M-dNTP, 4 μl cDNA, 0.2 M each primer, 1 \times polymerase chain reaction buffer and 1 U Taq polymerase. The conditions were 94 $^{\circ}\text{C}$ for 45 s, 65 $^{\circ}\text{C}$ for 45 s, 72 $^{\circ}\text{C}$ for 2 min and a final extension period of 7 min at 72 $^{\circ}\text{C}$. The amplification cycles were twenty-five for PS2 and Bcl-2, and nineteen for β -actin. The polymerase chain reaction products were separated on agarose (1.8 %) gel and stained with ethidium bromide. Separate experiments were performed to standardize the amount of RNA and the number of amplification cycles. The validation procedure was based on the linearity of the optical density reading scanned from the gel photographs.

Western analysis

Treated cells in triplicate were washed once with PBS (pH 7.4) and harvested into a 1.5 ml microtube with 0.5 ml lysis buffer (ml/l PBS: NP40 10, sodium deoxycholate 5 g, SDS 1 g). The lysis buffer contained protease inhibitors (l PBS: phenylmethylsulfonyl fluoride 40 mg, aprotinin 0.5 mg, leupeptin 0.5 mg, EDTA 1.1 mmol, pepstatin 0.7 mg). The harvested cells were then lysed with a Tekmar Sonic Disrupter (Cincinnati, OH, USA) on ice for 30 s. The protein concentration of cell lysate was determined by Dc protein assay (BioRad, Richmond, CA, USA). Lysate protein (25 μg) was separated on SDS (100 ml/l)-PAGE (Novex, San Diego, CA, USA) and transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA, USA). Anti-Bcl-x(L), anti-Bak, and anti-actin primary and secondary

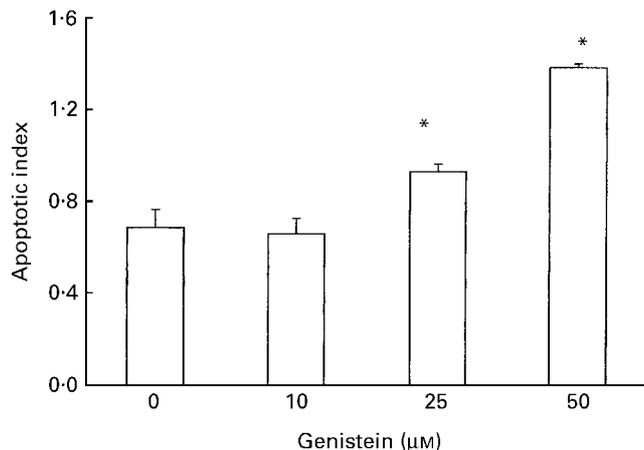


Fig. 1. Cell death induced by genistein: MCF-7 cells were seeded in twenty-four-well culture plates and treated with 0, 10, 25 or 50 μM -genistein for 48 h. A cell death ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany) was used to determine the DNA fragments and the values were normalized by cell viability absorbance readings. For details of procedures, see p. 464. Values are means for three determinations with standard errors shown by vertical bars. Mean values were significantly different from those of control wells: * $P < 0.05$.

antibodies conjugated with horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An ECL detection kit (Amersham, Arlington Heights, IL, USA) provided the chemiluminescence substrate for horseradish peroxidase and the targeted protein was visualized by autoradiography for 1–2 min and the amount was quantified by densitometry.

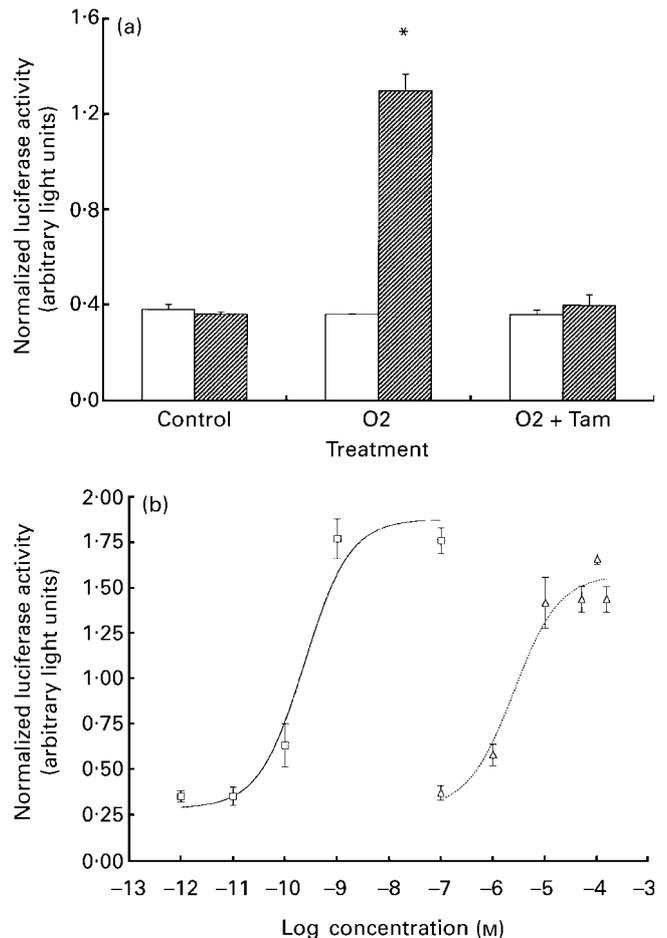


Fig. 2. Genistein-stimulated oestrogen receptor (OR)- α transactivation in HepG2 cells. HepG2 cells were cultured and transfected with oestrogen response element-luciferase, plasmid Renilla luciferase and OR- α expression or vector-only plasmids in twenty-four-well plates. The cells were lysed after 24 h treatment and the luciferase activities were measured. OR- α transactivation activity was measured as the firefly luciferase activity and normalized with the Renilla luciferase activity as the transfection control. For details of procedures, see p. 464. (a), The transfection of vector-only plasmid was used to show the functional expression of OR- α in HepG2 cells transfected with OR- α expression construct. O2, 17 β -oestradiol treatment; O2 + Tam, co-administration of 10 $^{-9}$ M-oestradiol and 10 $^{-7}$ M-tamoxifen. \square , Vector only; \blacksquare OR plasmid. Values are means for three determinations with standard errors shown by vertical bars. Mean value was significantly different from that of the control wells with no oestradiol treatment: * $P < 0.05$. (b), Various genistein and oestradiol concentrations were administered to the medium to obtain the dose–response relationships of the chemicals. \square , Oestradiol; Δ , genistein. Values are means for three determinations with standard errors shown by vertical bars; the points are curve-fitted by a built-in sigmoidal dose–response equation. The concentration that produced 50% of the maximum response (EC $_{50}$) of genistein is estimated as 2.5 (95% CI 0.4, 16.0) μM .

Statistical methods

A Prism® 3.0 software package (GraphPad Software Inc., San Diego, CA, USA) was utilized for statistical analysis. The results, whenever applicable, were analysed by one-way ANOVA followed by Dunnett's multiple comparison test if significant differences ($P < 0.05$) were observed. The OR transactivation luciferase activities and the OR competitive fluorescence readings were curved-fitted by sigmoidal dose-response built-in equations, which automatically calculated the concentration that produced 50% of the maximum response (EC_{50}) and the concentration that caused 50% of inhibition values (IC_{50}). The optical density results of Bak and Bcl-x(L) protein expressions were analysed by one-sample *t* test followed by Wilcoxon's signed rank test.

Results

Genistein-induced MCF-7 cell death

MCF-7 cells were treated with 0, 10, 25 and 50 μM -genistein for 48 h. The apoptotic indexes at 25 and 50 μM were significantly ($P < 0.05$) higher than those of the control cells (0 μM) as shown in Fig. 1. This indicated that a significant number of cells undergoing apoptosis in cultures treated with 25 and 50 μM -genistein.

The oestrogen receptor- α specific transactivation initiated by genistein

A validation assay was performed to verify the functionality of the OR- α plasmid. HepG2 cells were transfected with ORE-reporter plasmid, Renilla luciferase plasmid, and one of OR-expression plasmid or vector (plasmid

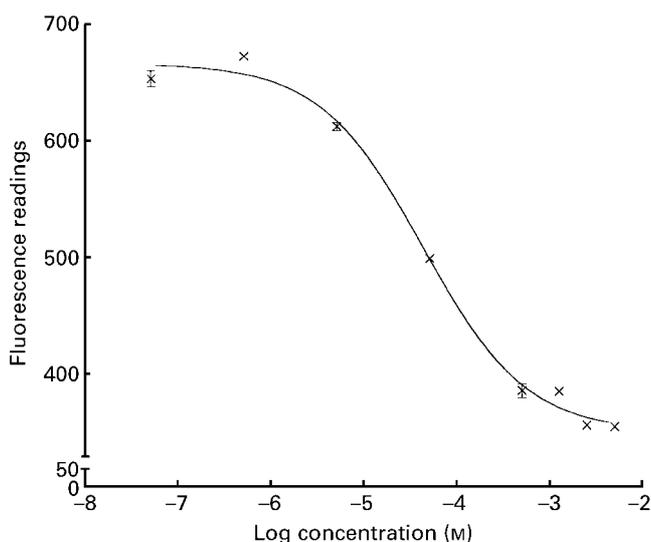


Fig. 3. The competition between genistein and 17 β -oestradiol for binding to oestrogen receptor (OR)- α . Various concentrations of genistein were mixed with 10⁻⁹M-fluorescein-labelled-17 β -oestradiol, and the mixture was then added to a plate coated with recombinant human OR- α . For details of procedures, see p. 464. Values are means for three determinations with standard errors shown by vertical bars. The concentration that caused 50% of inhibition (IC_{50}) is estimated as 48.0 (95% CI 12.0, 192.0) μM .

without OR- α insert). Cells transfected with vector only did not respond to oestradiol (10⁻⁹M) treatment, whereas cells transfected with OR expression plasmid demonstrated an increase in luciferase activity (Fig. 2(a)). The increased luciferase activity could be inhibited by tamoxifen (10⁻⁷M). The present experiment demonstrated that the OR plasmid was functional. Subsequently, experiments using the same system were carried out to examine genistein's effect on OR- α . Genistein displayed a dose-response oestrogenic effect on HepG2 cells expressing OR- α . The normalized luciferase activities indicated that genistein had an EC_{50} of about 2.5 μM , whereas the EC_{50} of 17 β -oestradiol was 0.23 nM (Fig. 2(b)). The results revealed that the OR- α transactivation by genistein was about 10 000-fold weaker than that of oestradiol.

The competition for oestrogen receptor- α binding

Having seen the difference in OR- α transactivation between genistein and oestradiol, the receptor binding affinity was also determined. Recombinant human OR- α receptors saturated with 10⁻⁹M-fluorescein-labelled-oestradiol were incubated with various concentrations of genistein. The concentration at which genistein out-competed half of the fluorescein-labelled oestradiol (IC_{50}) was estimated to be 48.0 μM (Fig. 3). In addition, we used unlabelled 17 β -oestradiol to correct for the possible interference of the fluorescein conjugate to the receptor, and estimated that the IC_{50} for the unlabelled oestradiol was 20 nM (results not shown). These results illustrated that genistein was about 2400-fold weaker than the unlabelled oestradiol for the affinity towards the ligand-binding domain of OR- α .

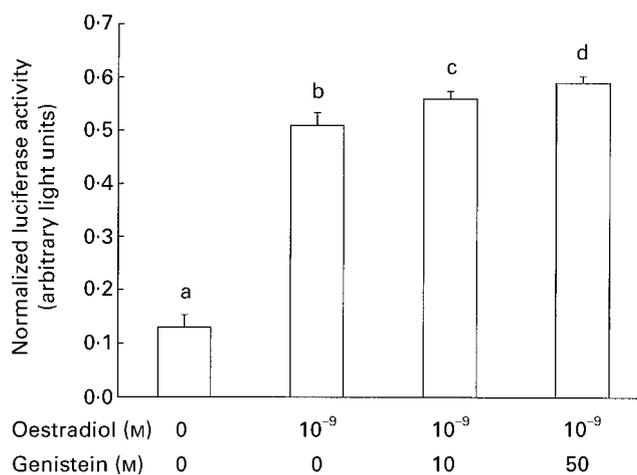


Fig. 4. Effect of genistein on oestradiol-induced oestrogen response (OR)- α transactivation in HepG2 cells. HepG2 cells were cultured and transfected with OR- α oestrogen response element-luciferase and Renilla luciferase plasmids in twenty-four-well plates. Genistein concentrations of 0, 10 or 50 μM were co-administered with 10⁻⁹M-17 β -oestradiol to the medium. The cells were lysed after 24 h treatment and the luciferase activities were measured. OR- α transactivation activity was measured as the firefly luciferase activity and normalized with the Renilla luciferase activity as the transfection control. For details of procedures, see p. 464. Values are means for three determinations with standard errors shown by vertical bars. Mean values are in the order: a < b < c < d ($P < 0.05$, ANOVA).

The interaction between genistein and 17 β -oestradiol on the oestrogen response element

In order to investigate the anti-oestrogenicity of genistein, HepG2 cells expressing OR- α were co-treated with 17 β -oestradiol and genistein. Instead of antagonizing the OR- α transactivation induced by oestradiol, 10 and 50 μ M-genistein slightly ($P < 0.05$) increased the transactivation activity (Fig. 4). These results demonstrated that genistein at these two concentrations did not impede the nuclear events initiated by oestradiol.

Presenilin 2 and Bcl-2 mRNA expression

Because the ORE-driven luciferase activities were increased by genistein in the presence or absence of 17 β -oestradiol, we investigated the transcriptional activities of two oestrogen-responsive genes in MCF-7 cells. Compared with the control (0 μ M-genistein), the reverse transcription-polymerase chain reaction results indicated that PS-2 and Bcl-2 mRNA expressions were up regulated by genistein at all concentrations tested (Fig. 5). A concentration of genistein of 25 μ M at which apoptotic induction was observed also induced the expressions. *ps2* and *bcl-2* have been widely accepted as oestrogen-responding genes (Ciocca & Elledge, 2000). These results agreed to the OR transactivation results presented in Fig. 2.

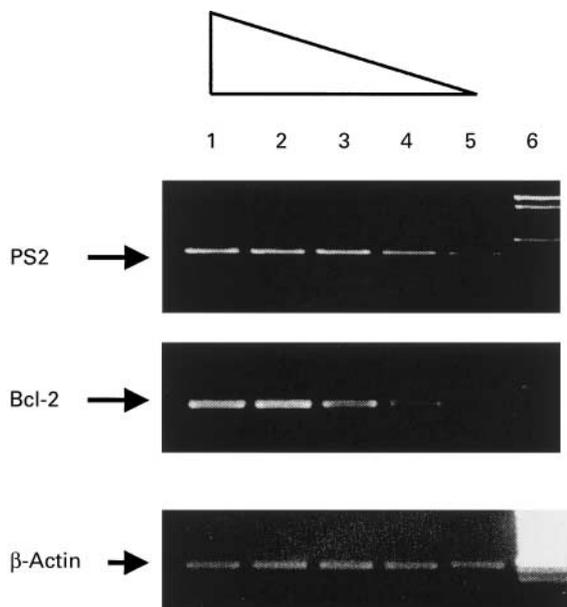


Fig. 5. Effect of genistein on the up-regulation of presenilin (PS) 2 and Bcl-2 mRNA expressions at concentrations leading to cell death. MCF-7 cells were treated with 0, 1, 10, 25 or 50 μ M-genistein for 48 h. Total RNA were isolated and semi-quantitative reverse transcription-polymerase chain reactions were performed to estimate the expressions of the oestrogen-responsive genes, PS2 and Bcl-2. Lanes 5, 4, 3, 2 and 1 correspond to 0, 1, 10, 25 and 50 μ M-genistein respectively, and lane 6 is a DNA marker. Each set represents one of two separate, independent experiments. For details of procedures, see p. 464.

Bak and Bcl-x(L) protein expressions affected by genistein

Since a previous study showed that Bcl-2 and Bak were both increased by genistein (Leung & Wang, 2000), we examined the role of the other Bcl-2 family members in the death process. Fig. 6 revealed that Bak and Bcl-x(L) protein expressions were differentially regulated by genistein at the concentrations which cell death was observed. Bak expression in cultures treated with 50 μ M-genistein was higher ($P < 0.05$) than the control (0 μ M-genistein), whereas Bcl-x(L) expression was decreased ($P < 0.05$) by 25 and 50 μ M-genistein.

Discussion

In the present study, we demonstrated that genistein potentiated OR transactivation with oestradiol administration, and correlation of OR antagonism and MCF-7

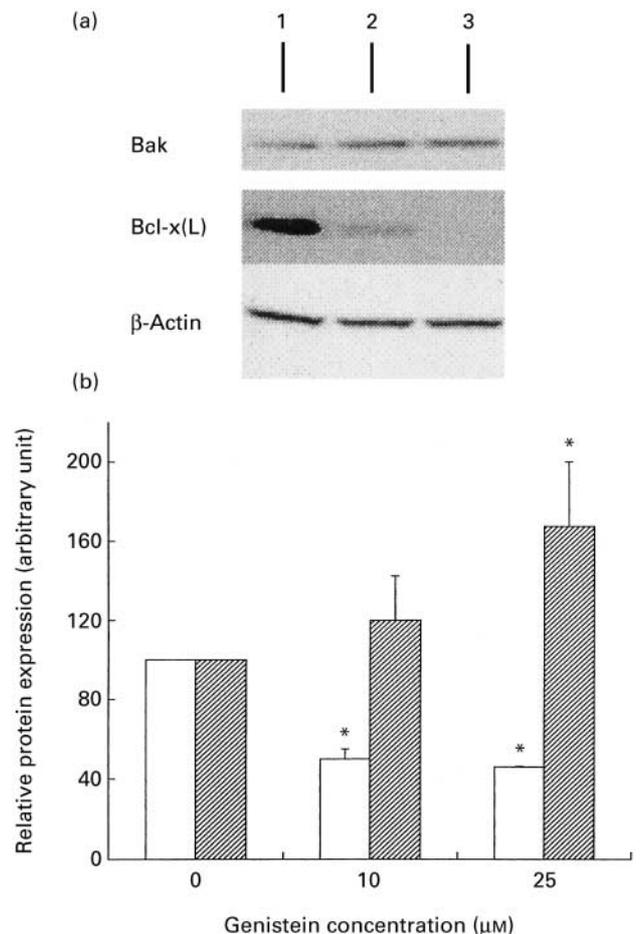


Fig. 6. Effect of genistein on Bak and Bcl-x(L) protein expressions. (a), Western analysis of MCF-7 cells treated with 0, 25 or 50 μ M-genistein (lanes 1, 2 and 3 respectively). The blot represents one of three separate, independent experiments; (b), optical densities of Bak and Bcl-x(L) proteins obtained in (a). β -Actin-normalized values are expressed as % control and are shown as mean values for three determinations with standard errors shown by vertical bars. \square , Bcl-x(L); ▨ , Bak. For details of procedures, see p. 464. Mean values were significantly different from those of the control wells: * $P < 0.05$.

cell death was not observed. It appeared the replacement of oestradiol by genistein as the ligand of OR did not deter the transactivation. When administered alone, genistein could increase PS2 and Bcl-2 mRNA expressions at the concentrations leading to cell death. These results suggested that genistein was a potentiator instead of an antagonist in the OR-initiated nuclear event at the death concentrations. On the other hand, Bak protein expression was up regulated while Bcl-x(L) was down-regulated by genistein at concentrations at which death was observed. These differential protein expressions favoured programmed cell death.

Many studies have shown that genistein is an agonist of OR, both in an animal model (Makela *et al.* 1995) or in cell culture models (Wang *et al.* 1996; Fioravanti *et al.* 1998; Miodini *et al.* 1999). Others studies more OR- α than normal breast tissue (Allred & Mohsin, 2000). The OR- α gene, however, is mostly lost in more advanced types of breast tumour (Sheng *et al.* 1996).

Recent studies have shown that genistein displays a proliferative effect at low concentrations (μM) (Wang & Kurzer, 1997; Breinholt & Larsen, 1998; Le Bail *et al.* 1998; Shao *et al.* 1998) and a cytotoxic effect at higher concentrations (Peterson & Barnes, 1991; So *et al.* 1997) in MCF-7 cells. However, the mechanism of cell death may not go through OR. A Bcl-2-independent pathway, for example the c-jun N-terminus kinase-mediated stress pathway, may be responsible for the apoptotic response of MCF-7 cells treated with 25 and 50 μM -genistein (Leung & Wang, 2000). That study that suggested that multiple pathways were involved in the action of genistein. At low concentrations, genistein reinforced ORE responses, which in turn might encourage proliferation in hormone-sensitive cells. Nonetheless, genistein could activate OR-independent pathways in the mediation of cell death as the dosage progressed.

The oestradiol and OR interaction can be multifaceted. There are at least four known oestradiol-initiated signalling pathways as reviewed by Hall *et al.* (2001). Two of them require the presence of ORE and others are ORE-independent. The transcriptional binding sites, AP-1, (Paech *et al.* 1997), Sp-1 (Batistuzzo de Medeiros *et al.* 1997), raloxifene response element (Yang *et al.* 1996) and genes containing the antioxidant response element (Montano & Katzenellenbogen, 1997) are all responsive to oestrogen administration. In the present study, we examined the classical ORE dependent pathways and found that genistein was not an effective antagonist of oestradiol. However, we could not rule out the possibility that genistein antagonized the ORE-independent signalling pathways. An *et al.* (2001) have demonstrated that genistein may

selectively generate an activation function-2 surface of OR- β that recruits co-regulators for the repression and activation of transcription. Since we did not examine the OR- β -mediated response in the present study, the effect of genistein on the downstream pathways of OR- β (either ORE dependent or independent) remain unknown. Considering the influences on the other promoters, it is still possible that genistein can be a pro- or anti-oestrogen, modulating transcription processes other than those dictated by ORE or OR- α .

The dietary relevance of the genistein dosages is not clearly established. Plasma concentrations of genistein ranging from $<1 \mu\text{M}$ (Arai *et al.* 2000) to $23 \mu\text{M}$ (Barnes, 1995) have been reported. Nevertheless, it appears that the classical anti-oestrogenic effect mediated by OR- α to be is unlikely a determining factor in cell death induced by genistein.

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