Different Effects of Genistein on Molecular Markers Related to Apoptosis in Two Phenotypically Dissimilar Breast Cancer Cell Lines

Jing Xu and George Loo*

Cellular and Molecular Nutrition Research Laboratory, Graduate Program in Nutrition, The University of North Carolina at Greensboro, Greensboro, North Carolina 27402-6170

Abstract   The association between consumption of genistein-containing soybean products and lower risk of breast cancer suggests a cancer chemopreventive role for genistein. Consistent with this suggestion, exposing cultured human breast cancer cells to genistein inhibits cell proliferation, although this is not completely understood. To better understand how genistein works, the ability of genistein to induce apoptosis was compared in phenotypically dissimilar MCF-7 and MDA-MB-231 human breast cancer cells that express the wild-type and mutant *p53* gene, respectively. After 6 days of incubation with 50 µM genistein, MCF-7 but not MDA-MB-231 cells, showed morphological signs of apoptosis. Marginal proteolytic cleavage of poly-(ADP-ribose)-polymerase and significant DNA fragmentation were also detected in MCF-7 cells. In elucidating these findings, it was determined that after 2 days of incubation with genistein, MCF-7 but not MDA-MB-231 cells, had significantly higher levels of p53. Accordingly, the expression of certain proteins modulated by *p53* was studied next. Levels of *p21* increased in both of the genistein-treated cell lines, suggesting that *p21* gene expression was activated but in a *p53*-independent manner, whereas no significant changes in levels of the pro-apoptotic protein, Bax, were found. In MCF-7 cells, levels of the anti-apoptotic protein, Bcl-2, decreased slightly at 18–24 h but then increased considerably after 48 h. Hence, the Bax:Bcl-2 ratio initially increased but later decreased. These data suggest that at the genistein concentration tested, MCF-7 cells in contrast to MDA-MB-231 cells were sensitive to the induction of apoptosis by genistein, but Bax and Bcl-2 did not play clear roles. J. Cell. Biochem. 82: 78–88, 2001.   © 2001 Wiley-Liss, Inc.

Key words: apoptosis; Bax; Bcl-2; breast cancer; genistein; p21; p53

Breast cancer is the second most common cause of death among all forms of cancer in women [Parker et al., 1997], which has made the prevention and treatment of breast cancer a major health issue and healthcare goal. Many of the present breast cancer chemopreventive and chemotherapeutic agents can have undesirable side effects. For example, treatment with the non-steroidal anti-estrogenic drug, tamoxifen, is associated with increased risk of endometrial cancer [Smith et al., 2000]. Therefore, there has been increasing interest in using natural alternative substances to prevent or treat breast

cancer without the side effects. In particular, genistein (5,7,4′-trihydroxyisoflavone), which is a phenolic phytochemical that is found in soybean products in high amounts, has been examined closely.

The suspicion of an anti-breast cancer effect of genistein comes from epidemiological studies [Nomura et al., 1978; Hirayama, 1981; Ingram et al., 1997, Wu et al., 1998] that have linked diets rich in soybean products with lower incidences of breast cancer. Furthermore, animal studies [Baggott et al., 1990; Barnes et al., 1990] have established that feeding soy-based diets to rats reduces chemical carcinogen-induced formation of mammary tumors. Taken together, these findings have led to the general speculation that genistein is the primary soybean phytochemical that has anti-breast cancer activity. However, to advance the notion of genistein as a potential breast cancer chemopreventive agent, cellular studies have been

conducted as well. For example, genistein inhibited the growth of cultured human breast cancer cells [Fioravanti et al., 1998; Shao et al., 1998]. This inhibitory effect of genistein is attributed to the induction of cell cycle arrest and/or apoptosis, although full characterization of the molecular events has not been completed.

Apoptosis is essential for normal physiological development but also critical in eliminating any abnormal cells that result after exposure to genotoxic or DNA-damaging agents. In response to the DNA damage, levels of p53 tumor suppressor protein become elevated which triggers cell cycle arrest to permit DNA repair. On the other hand, if the DNA damage is irreparable, the genetic program for apoptosis is activated, and eventually, the affected cells are eliminated in an orderly and controlled manner [Ding and Fisher, 1998]. These basic phenomena have been exploited in devising strategies to kill cancer cells, thus providing the basis of some forms of cancer chemotherapy [Fisher, 1994].

Previously, it was demonstrated that genistein induced protein-linked DNA damage in leukemia cells, [Markovits et al., 1989]. More recently, it was reported that genistein induced DNA damage or strand breakage in colon cancer cells [Salti et al., 2000]. It is thought that genistein-induced DNA damage is due to inhibition of DNA topoisomerase II, which is known to result in double-strand breaks in DNA [Malonne and Atassi, 1997]. Therefore, it is conceivable that such DNA damage induced by genistein can initiate multiple events leading to apoptosis. That is, there may be elevation in the levels of p53, which is a transcription factor for numerous genes including some involved in apoptosis [Fields and Jang, 1990]. For example, *WAF1(p21)* [El-Deiry et al., 1993], which halts cell cycle progression by inhibiting cyclin-dependent kinases, and *Bax* [Miyashita and Reed, 1995], which promotes apoptosis, are two genes that are transcriptionally activated by p53. On the other hand, p53 suppresses the transcription of other genes [Mack et al., 1993] such as *Bcl-2* [Miyashita et al., 1994; Budhram-Mahadeo et al., 1999], which is an anti-apoptotic factor. Accordingly, increased levels of p21 protein would stimulate cell cycle arrest that may be followed by apoptosis, which typically happen when cells sustain DNA damage [El-Deiry et al., 1994]. Then too, the subsequent increase in Bax and decrease in Bcl-2 protein levels would promote apoptosis [Oltvai et al., 1993]. Other apoptotic events might also occur such as caspase-mediated cleavage of the DNA repair enzyme, poly-(ADP-ribose)-polymerase (PARP) [Kaufmann et al., 1993; Duriez et al., 1997], and fragmentation of DNA [Pandey et al., 1994] by a caspase-activated deoxyribonuclease [Enari et al., 1998].

Because genistein induces DNA double-strand breaks [Markovits et al., 1989; Salti et al., 2000] which could lead to p53-mediated apoptosis, we compared the capacity of genistein to induce apoptosis in phenotypically dissimilar MCF-7 and MDA-MB-231 human breast cancer cells that express the wild-type and mutant *p53* gene, respectively. We determined if the *p53* gene status of human breast cancer cells influences the ability of genistein to induce the expression of several relevant molecular markers related to apoptosis.

MATERIALS AND METHODS

Genistein, camptothecin, doxorubicin, ethidium bromide, normal and low melting point agarose products, trypsin, Eagle's minimum essential medium and Leibovitz's L-15 medium were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from BioWhittaker, Inc. (Walkersville, MD). Antibiotics were purchased from Mediatech, Inc. (Herndon, VA) as a stock solution (10,000 U/ml penicillin G, 10,000 μg/ml streptomycin, 25 μg amphotericin B). MCF-7 and MDA-MB-231 human breast cancer cell lines were purchased from American Type Culture Collection (ATCC) (Rockville, MD). These distinct cell lines, which are adherent and have epithelial morphology when cultured, originated from separate mammary gland adenocarcinomas present in two different female Caucasians. The cells were obtained from the adenocarcinomas of the human subjects by pleural effusion. As characterized by others [Elstner et al., 1995; Liu et al., 1997], MCF-7 cells express estrogen receptors and wild-type *p53* gene and are also minimally invasive. MDA-MB-231 cells are estrogen receptor-negative, expressive mutant *p53* gene, and are highly invasive or metastatic.

SDS-PAGE mini-gels (4–20% Tris-Glycine), See-Blue prestained protein markers, running buffer (10x) were purchased from Novex (San Diego, CA). The Hybond ECL nitrocellulose

membrane was purchased from Amersham Life Science (Arlington Heights, IL). The mouse monoclonal anti-*p53* antibody (clone BP53-12) was purchased from Sigma Chemical Co. (St. Louis, MO). The mouse anti-*WAF1 / p21* monoclonal antibody (clone EA10) and rabbit anti-*bax* polyclonal antibody were purchased from Oncogene Research Products (Cambridge, MA). The mouse anti-*bcl-2* antibody (clone BCl-2-100) was purchased from Zymed Laboratories Inc. (San Francisco, CA). The rabbit anti-PARP polyclonal antibody (clone VIC5) was purchased from Roche Molecular Biochemicals (Indianapolis, IN).

Cell Culture

MCF-7 and MDA-MB-231 cells were routinely maintained in complete Eagle's minimum essential medium and Leibovitz's L-15 medium, respectively, as recommended by ATCC. The cell culture media were each supplemented with 10% FBS, 1% penicillin G/streptomycin, and 0.2% amphotericin B. MCF-7 cells were cultured at $37^{o}C$ in a humidified atmosphere of 95% air/5% CO_2 and subcultured once a week at a ratio of 1:5. MDA-MB-231 cells were cultured at $37^{\circ}C$ in a humidified atmosphere of 100% air and subcultured once a week at a ratio of 1:25. Cell culture medium was renewed 2–3 times/ week.

Morphological Examination of Cells After Treatment With Genistein

On the second day after seeding, MCF-7 and MDA-MB-231 cells (1×10^6 cells/ml) were incubated for 0–72 h with 0 and 50 μM of genistein dissolved in DMSO. The final concentration of DMSO was 0.1% in both genistein-treated and untreated control cells. The cells were examined with an Olympus IX-70 inverted microscope equipped with PM-C35DX camera.

Detection of Poly-(ADP-Ribose)-Polymerase (PARP) Cleavage in Cells After Treatment With Genistein

MCF-7 (7.5×10^5 cells/10 ml) and MDA-MB-231 cells (1.5×10^5 cells/10 ml) were incubated for 0–12 days with 50 μM of genistein dissolved in DMSO. Cells treated with 0.1% DMSO were used as a negative vehicle control. MCF-7 cells treated with 0.4 μg/ml camptothecin for 24 h were used as a positive control. All cells were treated on the second day after seeding.

To harvest the cells, they were washed with Ca^{2+}/Mg^{2+}-free Hank's Balanced Salt Solution (HBSS) once and detached with 0.25% trypsin/ 0.03% EDTA in HBSS. After trypsinization, cells were pelleted at $300 \times$ g for 10 min, and the cell pellets were washed with phosphate buffered saline (PBS) once. Then, the cells were suspended in lysis buffer (50 mM KCl, 0.5% NP-40, 25 mM HEPES (pH7.8), 1 mM PMSF, 10 μg/ ml leupeptin, 20 μg/ml aprotinin, 100 μM DTT) and left on ice for 4 min. The above cell lysates were centrifuged at 16,000g for 1 min. The nuclei-containing cell pellets were washed once with buffer (50 mM KCl, 25 mM HEPES (pH7.8), 1 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μM DTT) and then resuspended in nuclear extraction buffer (500 mM KCl, 10% glycerol, 25 mM HEPES (pH 7.8), 1 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μM DTT) and left on ice for 30 min. After centrifugation, the supernatant was collected and saved as the nuclear extract.

The proteins in the nuclear extract (50 μg) were separated by SDS-PAGE and blotted to a nitrocellulose membrane. The nitrocellulose membrane containing the transferred proteins was then blocked with 5% non-fat milk. Next, the membrane was probed with rabbit anti-PARP primary antibody and corresponding goat anti-rabbit secondary antibody conjugated to horseradish peroxidase. The nitrocellulose membrane was then incubated with chemiluminescent developing reagent for 1 min and then exposed to X-ray film. The presence of PARP protein and cleaved products was then visualized by developing the film.

Determination of DNA Fragmentation in Cells After Treatment With Genistein

MCF-7 (7.5×10^5 cells/10 ml) and MDA-MB-231 cells (1.5×10^5 cells/10 ml) were treated for 72 h with 50 μM of genistein dissolved in DMSO. Cells treated with 0.1% of DMSO were used as a negative vehicle control. MCF-7 and MDA-MB-231 cells treated with 0.4 μg/ml camptothecin for 24 h were used as a positive control. All cells were treated on the second day after seeding.

After treatment, cells were detached by trypsinization and harvested. DNA fragmentation was determined using the Cell Death Detection ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN). Briefly, 5×10^4 cells/sample were suspended for 30 min on ice in 500 μl of solubilization buffer, which destroys

the plasma membrane of cells without disturbing the nuclear membrane. Afterwards, the lysate was centrifuged at $16,000g$ at 4°C for 10 min. The supernatant, which contains any low molecular weight DNA fragments resulting from apoptosis, was collected. To quantify the DNA fragments, a microtiter plate was first coated with anti-histone antibody and then incubated with the supernatant to allow binding of any mono- and oligonucleosomes to the anti-histone antibody. Next, the microtiter plate was incubated with anti-DNA-peroxidase-labeled antibody, which binds to the DNA component of the nucleosomes, thus forming immunocomplexes that were quantified by incubating next with a peroxidase substrate (ABTS). The resulting absorbance at 405 nm against 492 nm were measured using a TECAN SLT 340 ATTC microtiter plate reader.

Determination of p53, p21, bax, and bcl-2 Protein Expression in Cells After Treatment With Genistein

MCF-7 (7.5×10^5 cells/10 ml) and MDA-MB-231 cells (1.5×10^5 cells/10 ml) were treated for 0–9 days with 50 µM of genistein dissolved in DMSO. Cells treated with 0.1% of DMSO were used as a negative vehicle control. MCF-7 cells treated with 0.2 µg/ml doxorubicin for 48 h were used as a positive control. All cells were treated on the second day after seeding.

To harvest the cells, they were washed with HBSS once and detached with 0.25%trypsin/ 0.03% EDTA in HBSS. After trypsinization, cells were pelleted at $300 \times g$ for 10 min, and the cell pellets were washed with PBS once. Then, the cells were suspended in lysis buffer (25 mM Tris Cl (pH8.0), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 100 µg/ml PMSF, 1 mM Na_3VO_4, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 100 µM DTT) and left on ice for 30 min. Next, the above cell lysates were centrifuged at $16,000g$ for 10 min. The supernatant was collected as the whole cell lysates.

The proteins in the whole cell lysates (50 µg) were separated by SDS-PAGE and blotted to nitrocellulose membrane. The membrane was then blocked with non-fat milk and probed with rabbit anti-bax primary antibody (1:200) and the corresponding secondary antibody (1:5000) conjugated to horseradish peroxidase. Next, the nitrocellulose membrane was incubated with the chemiluminescent developing reagent for 1 min and then exposed to X-ray film. The presence of bax protein was then visualized by developing the film. Afterwards, the same membrane was stripped with buffer (62.5 mM Tris Cl (pH6.8), 2% w/v SDS, 100 mM β-mercaptoethanol) at 55°C for 30 min. The stripped membrane was reprobed with mouse anti-bcl-2 antibody (1:1000) and the corresponding secondary antibody (1:5000) to assess the intracellular bcl-2 levels using the same procedure described above. In this way, the same membrane was stripped and reprobed with mouse anti-p21 antibody (1:100), mouse anti-p53 antibody (1:2000), rabbit anti-actin antibody (1:200), and mouse anti-tubulin antibody (1:200). Levels of protein expression were quantified using a Bio-Rad GS-670 Imaging Densitometer.

Data Analysis

Data were analyzed by the one-way analysis of variance Tukey HSD multiple comparison test using the Systat software v9.01 (SPSS Inc., Chicago, IL).

RESULTS

To determine if exposing human breast cancer cells to genistein results in morphological changes characteristic of apoptosis, MCF-7 and MDA-MB-231 cells were incubated with 0 and 50 µM genistein for 0–6 days. After 4 days of incubation, cell shrinkage and membrane blebbing were seen in only a limited population of the genistein-treated MCF-7 cells. But, after 6 days of incubation (Fig. 1), the majority of the MCF-7 genistein-treated cell populations showed such morphological features of apoptosis. Moreover, the majority were detached from the culture dishes, although a small number of these cells were still attached and apparently viable. These viable cells were larger in size than the control cells, and they also had increased intracellular vacuoles upon closer examination. Thus, these visual observations suggest that genistein induced apoptosis in most of the individual MCF-7 cells, but differentiation in the remainder of the cells.

On the other hand, most of the genistein-treated MDA-MB-231 cells were still viable, but much larger in size than untreated cells after 6 days of incubation (Fig. 1). Even after 12 days of exposure to genistein, no appreciable morphological signs of apoptosis were seen (data not shown). Instead, more than one nucleus was
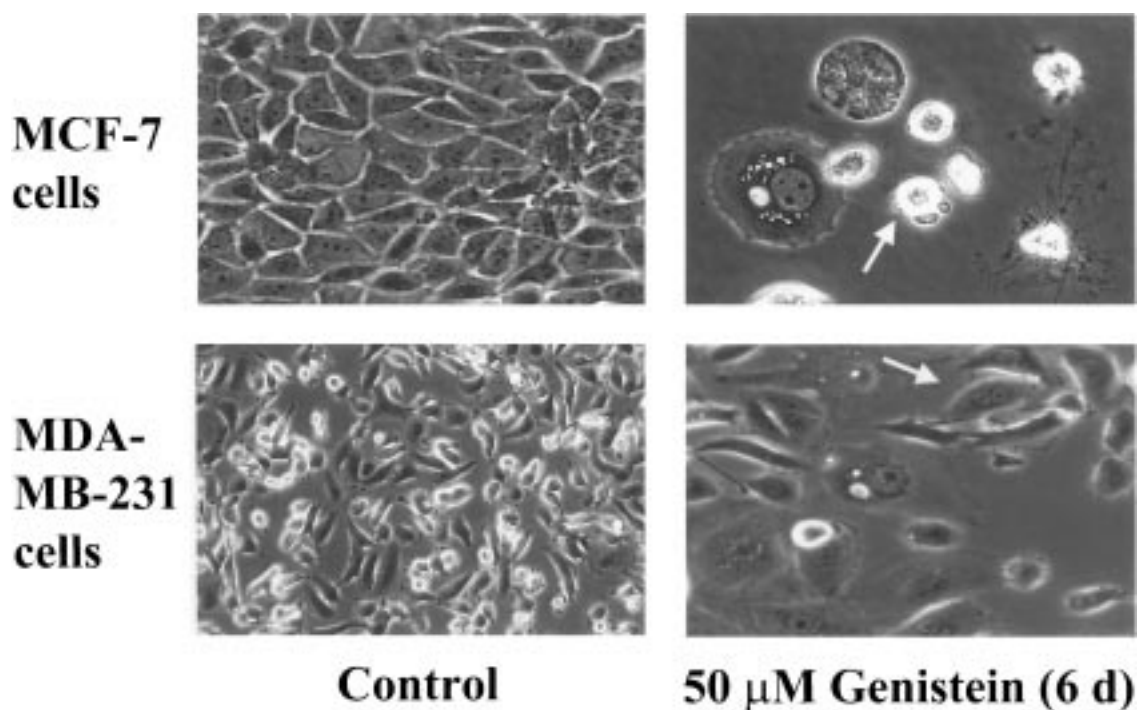
Fig. 1. Morphology of MCF-7 and MDA-MB-231 human breast cancer cells after incubation with either 0 (control) or 50 µM genistein for 6 days. All photos were taken at 200X magnification. Arrow in upper-right photo; an apoptotic cell with cell shrinkage and membrane blebbing. Arrow in lower-right photo; a cell with two nuclei.

seen in many of the cells, which is a similar feature seen in some normal mammalian cells that may also be polyploidal [Mowat and Stewart, 1998]. The nuclei were normal or slightly smaller in size and located mainly in the middle of the cells. In contrast, the cytoplasm was more flat and expanded in area. Thus, these visual observations suggest that genistein induced differentiation but not apoptosis in MDA-MB-231 cells.

To examine other markers of apoptosis, proteolytic cleavage of the nuclear protein, PARP, was assessed by SDS-PAGE/Western-immunoblotting analysis of MCF-7 and MDA-MB-231 cells that had been incubated with 50 µM genistein for 0–6 days (Fig. 2). In the positive control (camptothecin-treated cells), PARP cleavage was detected in both MCF-7 and MDA-MB-231 cells as indicated by the presence of an 89 kD band that reflects a proteolytic fragment of the 116 kD PARP protein. Unexpectedly, in the case of MDA-MB-231 cells, slight presence of the 89 kD proteolytic fragment was detected in the untreated control and also DMSO-treated vehicle control. Moreover, genistein did not induce

PARP cleavage since there was no increase in band intensity of the 89 kD fragment. In the case of MCF-7 cells, where the 89 kD fragment was not detected in the untreated control and also DMSO-treated vehicle control, genistein induced only slight PARP cleavage as indicated by the presence of the 89 kD fragment after 4 days of incubation.

As the results of the PARP cleavage assay were not absolutely clear in assessing apoptosis, the Cell Death Detection ELISA was performed on both cell lines that had been exposed to 50 µM genistein for 72 h to further assess apoptosis. The assay detects endonuclease-mediated DNA fragmentation, a common event in apoptosis. As shown in Figure 3, genistein significantly increased DNA fragmentation in MCF-7 cells as indicated by the greater optical density readings relative to that of control cells. In contrast, genistein did not produce such an effect in MDA-MB-231 cells.

Having established to a reasonable extent that genistein induced apoptosis at least in MCF-7 cells, we were prompted next to examine the expression of p53 and p21 proteins by Western analysis. Genistein had different
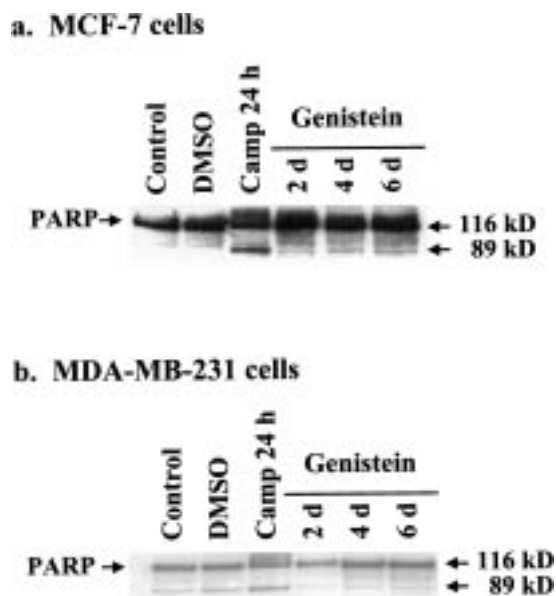
a. MCF-7 cells

b. MDA-MB-231 cells

Fig. 2. Detection of proteolytic cleavage of poly (ADP-ribose) polymerase (PARP) in MCF-7 and MDA-MB-231 cells after incubation with genistein. MCF-7 cells (**A**) and MDA-MB-231 cells (**B**) were incubated with 50 µM genistein for 2–6 days. Cells treated with 0.1% DMSO were used as a vehicle control. Cells treated with 0.4 µg/ml camptothecin (Camp) were used as a positive control. Nuclear extracts were assayed, and any proteolytic cleavage of PARP was detected by SDS-PAGE/Western-immunoblotting analysis. Cleavage of PARP (116 kD) to an 89 kD fragment is indicative of apoptosis.

effects in the two cell lines. In MCF-7 cells incubated with 50 µM genistein, *p53* expression increased slightly at 2 h, peaked at 48–72 h, and then dropped at 6 day of incubation (Fig. 4). In contrast, there were no significant changes in the expression of *p53* in MDA-MB-231 cells
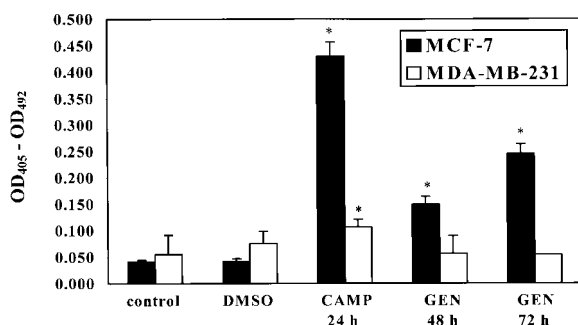

Fig. 3. Determination of DNA fragmentation in MCF-7 and MDA-MB-231 cells after incubation with 50 µM genistein (GEN). Cells treated with 0.1% DMSO were used as a vehicle control. Cells treated with 0.4 µg/ml camptothecin (CAMP) were used as a positive control. After 24–72 h of incubation, the extent of DNA fragmentation was determined by the Cell Death Detection ELISA as described under Materials and Methods.

throughout the entire incubation period. Consistent with the increased expression of *p53*, genistein-treated MCF-7 cells also had increased expression of *p21*. Highest expression was found after 48 h of incubation with genistein with only a slight decline occurring over the next 4 days. Despite no change in the expression of *p53* in MDA-MB-231 cells, increased expression of *p21* was detected after only 18 h of incubation with genistein and increased further with continued incubation.

To consider if genistein induced apoptosis via *p53*-dependent modulation of *Bax* and *Bcl-2* gene expression, we finally examined the expression of Bax and Bcl-2 protein levels in MCF-7 and MDA-MB-231 cells that had been incubated with 50 µM genistein (Fig. 5). There were no significant changes in *Bax* expression in either cell line during the entire incubation period. In MCF-7 cells, the expression of *Bcl-2* decreased slightly after 18–24 h, but then increased noticeably after 48 h of incubation with genistein. In the genistein-treated MDA-MB-231 cells, *Bcl-2* expression increased slightly overall. Densitometric analysis of the expression of *Bax* and *Bcl-2* as a ratio is presented in Figure 6. In MCF-7 cells, the Bax:Bcl-*2* ratios increased at 18–24 h of incubation with genistein, but then dropped to ratios lower than that in the control cells. In contrast, the Bax:Bcl-2 ratios in genistein-treated MDA-MB-231 cells were lower than that of the control cells.

DISCUSSION

Complete characterization or full elucidation of how genistein inhibits the proliferation of cultured human breast cancer cells has not been achieved yet. However, a partial explanation of the anti-proliferative effect of genistein is that this phytochemical kills the cells by inducing apoptosis. Several research groups [Constantinou et al., 1998; Shao et al., 1998; Li et al., 1999] have reported that incubating human breast cancer cells with genistein results in apoptosis. As an expansion of such previous work, our data collectively indicated that genistein induce apoptosis in MCF-7 cells but not in MDA-MB-231 cells. This difference in sensitivity of the two cell lines to genistein might reflect their dissimilar phenotypes. For example, as described elsewhere [Elstner et al., 1995; Liu et al., 1997], MCF-7 cells express estrogen receptors
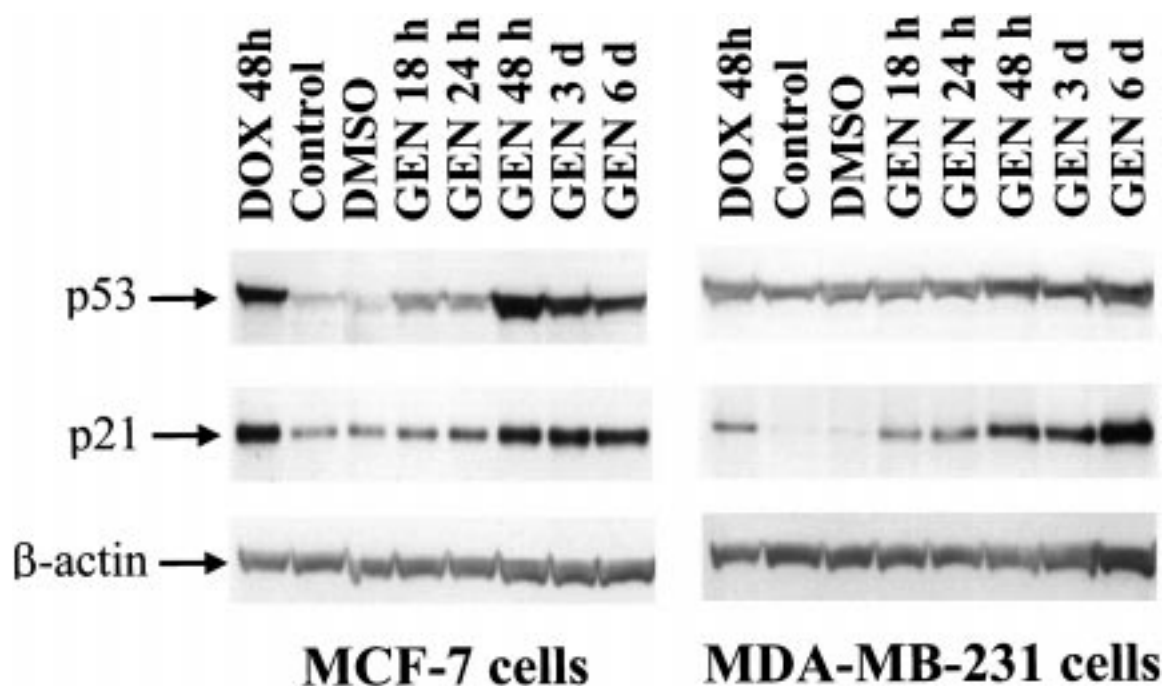
Fig. 4. Effects of genistein (GEN) on the expression of p53 and p21 protein in MCF-7 and MDA-MB-231 cells. Cells were incubated for 18–6 days with 50 µM GEN. Cells treated with 0.1% DMSO were used as a vehicle control. Cells treated with 0.5 µg/ml doxorubicin (DOX) were used as a positive control. The expression of p53, p21 and β-actin was determined by SDS-PAGE/Western-immunoblotting analysis as described under Materials and Methods.

and wild-type *p53* gene and are also minimally invasive. MDA-MB-231 cells are estrogen receptor-negative, expressive mutant *p53* gene, and are highly invasive.

In assessing apoptosis in the present study, morphological examination of the cells by light microscopy and analysis of DNA fragmentation by the Cell Death Detection ELISA provided
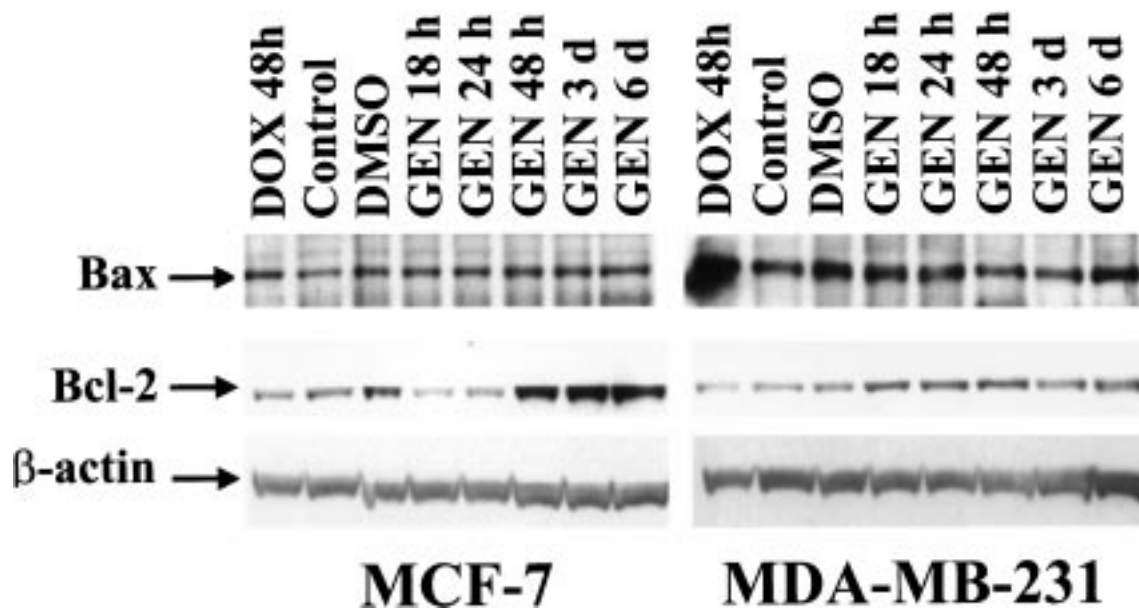

Fig. 5. Effects of genistein (GEN) on the expression of Bax and Bcl-2 protein in MCF-7 and MDA-MB-231 cells. Cells were incubated for 18 h–6 days with 50 µM GEN. Cells treated with 0.1% DMSO were used as a vehicle control. Cells treated with 0.5 µg/ml doxorubicin (DOX) were used as a positive control. The expression of Bax, Bcl-2 and β-actin was determined by SDS-PAGE/Western-immunoblotting analysis as described under Materials and Methods.
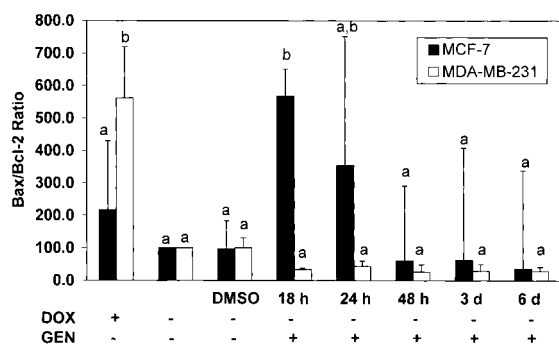
Fig. 6. Effects of genistein (GEN) on the Bax:Bcl-2 ratio in MCF-7 and MDA-MB-231 cells. Cells were incubated for 18 h– 6 days with 50 µM GEN. Cells treated with 0.1% DMSO were used as a vehicle control. Cells treated with 0.2 µg/ml doxorubicin (DOX) were used as a positive control. The expression levels of Bax and Bcl-2 protein were quantified by densitometry, and from the arbitrary units the Bax:Bcl-2 ratios were then calculated.

evidence of apoptosis in MCF-7 cells that had been incubated with 50 µM genistein for 6 days. The detection of proteolytic cleavage of PARP, which is recognized as an early marker of apoptosis, in genistein-treated MCF-7 cells was not quite successful or revealing. Using nuclear extracts, only marginal cleavage of the nuclear protein, that is involved in DNA repair, i.e., PARP, was detected. Nevertheless, we also observed chromatin condensation after DAPI staining of MCF-7 cells that had been incubated with 50 µM genistein for 6 days (data not shown). Among all the methods for the detection of apoptosis, cell morphological changes, especially chromatin condensation, are considered the most accurate indicators of apoptosis [Willingham, 1999]. Thus, in the present study, evaluating several standard markers allowed better assessment of apoptosis.

By comparison, our results are in partial agreement with that of a similar study with MCF-7 cells [Constantinou et al., 1998], where within 2 days genistein induced differentiation at concentrations of 10–45 µM, but triggered apoptosis at much higher concentrations of 100–300 µM. We saw MCF-7 cells initially undergoing differentiation after 48–72 h of incubation with 50 µM genistein, as indicated by the appearance of intracellular neutral lipid droplets and an increase of intracellular casein levels (data not shown). However, upon continued incubation with 50 µM genistein, markers of apoptosis appeared at Day 4, but at Day 6, the majority of the MCF-7 cells were affected. Thus, our findings have revealed that a lower

concentration of genistein (i.e., 50 µM rather than 100–300 µM) is capable of inducing apoptosis in MCF-7 cells if the time of incubation is prolonged. This is more relevant to physiological conditions where small amounts of genistein in soybean products are ingested in the diet for a long period of time to exert a chronic health effect.

Since apoptosis is often accompanied by increased expression of *p53* that is thought to actually mediate the apoptosis [Ding and Fisher, 1998], the expression of p53 protein and also p21 protein resulting from *p53*-dependent and *p53*-independent pathways was examined in the genistein-treated cells. Although genistein increased the expression of *p53* in only MCF-7 cells, there was increased expression of *p21* in both MCF-7 cells expressing wild-type *p53* gene and MDA-MB-231 cells expressing mutant *p53* gene. The *p53* gene in MDA-MB-231 cells is deleted in one allele and mutated at lys^{280} in exon 8 in the other allele [Runnebaum et al., 1994], resulting in non-functional p53 protein. Therefore, our findings suggest that genistein increases the expression of p21 protein in MDA-MB-231 cells via a *p53*-independent pathway. However, the possibility cannot be ruled out that the increased expression of p-21 protein in genistein-treated MCF-7 cells could be attributed either to *p53*-dependent or *p53*-independent pathways, or perhaps both. The *p21* gene promoter region contains multiple response elements for the binding of numerous transcription factors [Gartel and Tyner, 1999] and subsequent activation of the gene. While *p53* is important for *p21*-mediated events, this is not always the case. For example, the *p53*-dependent activation of *p21* gene transcription is critical for cellular responses to DNA damage, Neu differentiation factor, or ribonucleotide inhibitors. On the other hand, the *p53*-independent activation of *p21* gene transcription occurs in cells exposed to various agents that stimulate differentiation. These differentiation agents induce binding of transcription factors to specific cis-acting responsive elements, such as Sp1-3, Ap2, BRCA1, VDR, RAR, C/EBPαand β, STAT1, STAT3, and STAT5, in the promoter region of the *p21* gene. Hence, it is conceivable that genistein induced *p53*-independent expression of the *p21* gene by activating one or several of the transcription factors that bind to these response elements. It must also be considered that regulation of *p21* gene expression occurs at

multiple levels, including transcription and stabilization of *p21* mRNA [Shao et al., 1998], translation of *p21* mRNA [Gudas et al., 1995], and post-translational stabilization of p21 protein [Timchenko et al., 1996].

It is uncertain whether the increased expression of *p21* is essential for the apoptosis induced in MCF-7 cells by genistein. As a universal inhibitor of cyclin-dependent kinases (Cdk), *p21* predominately inhibits Cdk2 and Cdk4 during cell cycle arrest at the G_1/S phase transition [Harper et al., 1995]. Additionally, *p21* binds to proliferating cell nuclear antigen (PCNA) and blocks ongoing DNA synthesis and replication at the S phase [Flores-Rozas et al., 1994]. Rapid induction of *p21* gene expression and cell cycle arrest can result when cells are starved of serum or exposed to ultraviolet radiation and other DNA-damaging agents [Harada and Ogden, 2000]. Upon DNA damage, activation of *p21* gene expression that promotes cell cycle arrest at the G_1/S phase allows cells the opportunity to repair the damage. If DNA repair is successful, cell cycle progression will resume. If there is irreparable DNA damage, cells will undergo apoptosis. On the other hand, although *p21* is important for apoptosis in some systems [Duttaroy et al., 1997], this is not always true [Gorospe et al., 1997]. Moreover, cells lacking *p21* appear to undergo apoptosis normally [Brown et al., 1997]. In any event, a recent study suggests that cleavage of *p21* by caspases may be required to permit cells in a state of cell cycle arrest to commit apoptosis [Zhang et al., 1999]. Thus, *p21* may represent a unique intracellular sensor for making decisions regarding proliferation, cell cycle arrest, apoptosis, or differentiation. Genistein seems to influence all four of these processes in human breast cancer cells.

Increased expression of *p21* is also found when cells undergo G_2/M phase arrest and upon induction of differentiation [Dulic et al., 1998; Zhang et al., 1999]. In our study, the increased expression of p21 protein was much higher in MDA-MB-231 cells than in MCF-7 cells. This finding is consistent with our other recent work (unpublished data), where genistein induced cell cycle arrest at the G_2/M phase in both cell lines, but to a greater extent in MDA-MB-231 cells. Even though both MCF-7 and MDA-MB-231 cells exposed to genistein had increased expression of p21 protein and were arrested at the G_2/M phase of the cell cycle, MCF-7 cells

committed apoptosis whereas MDA-MB-231 cells differentiated. It is unclear why the increased *p21* expression had different end effects.

Since the expression of *p53* was increased by genistein in our MCF-7 cells, other *p53*-responsive genes besides *p21* may be affected as well. The *Bax* gene is transcriptionally activated [Miyashita and Reed, 1995] and the *Bcl-2* gene is transcriptionally suppressed [Miyashita et al., 1994; Budhram-Mahadeo et al., 1999] by *p53*. Nevertheless, the apoptosis in genistein-treated MCF-7 cells did not seem to involve the pro-apoptotic protein, Bax, because there was no significant change in the expression of Bax protein as determined by SDS-PAGE/Western-immunoblotting analysis. Unexpectedly, expression of the anti-apoptotic protein, Bcl-2, decreased in MCF-7 cells after 18–24 h of incubation with genistein, but then increased considerably after 48 h. The increased expression of *Bcl-2* would not be anticipated to promote apoptosis in the MCF-7 cells, but increased expression of Bcl-2 protein and apoptosis have been reported in normal human fibroblasts exposed to H_2O_2 [Chen et al., 2000]. Moreover, although an increased Bax:Bcl-2 ratio would be expected to promote apoptosis, we found only a transient increase of the Bax:Bcl-2 ratio after 18–24 h of incubation of MCF-7 cells with genistein that stimulated apoptosis. Later, the Bax:Bcl-2 ratio in these genistein-treated cells declined to a lower value than in control cells after 48 h of incubation. It is possible that the transient increase of the Bax:Bcl-2 ratio commits the genistein-treated MCF-7 cells to apoptosis, but after the death signal is generated, the ratio does not need to be sustained for the execution stage of apoptosis.

Besides *Bax* and *Bcl*-2, other *Bcl-2* family members such as $Bcl-X_L$, *Bcl-w*, *Bak*, and $Bcl-X_s$ may have also been affected in MCF-7 cells exposed to genistein. Thus, the overall ratio of the death agonists to *Bcl-2* family of antagonists could be more critical in determining the ultimate fate of the MCF-7 cells. Additionally, the apoptosis in MCF-7 cells could be via a "*Bcl-2*-insensitive" apoptotic pathway such as that triggered by tumor necrosis factor (TNF) or Fas ligand [Strasser et al., 2000]. *Bcl-2* may have a second function that is distinct from its ability to block apoptosis, as suggested by other research. Overexpression of *Bcl-2* promotes the exit of stressed cells from the cell cycle and also retards

quiescent G_0-phase cells from re-entering the cell cycle in response to mitogenic stimulation [Strasser et al., 1997]. Therefore, the increased expression of Bcl-2 protein in our genistein-treated MCF-7 cells may help prevent cells from re-entering the cell cycle.

While this work was in progress, it was reported [Li et al., 1999] that 30 μM genistein dissolved in 0.1 M Na_2CO_3 induced apoptosis, increased Bax and decreased Bcl-2 protein expression in MDA-MB-231 cells. These findings are obviously different than our findings. The discrepancies may be due to the use of different solvents to dissolve the genistein for incubation with the cells. We used the traditional solvent, DMSO, to give a final concentration of 0.1% in the culture media, which did not exert any effects on the measurement parameters. Nevertheless, we did expose our MDA-MB-231 cells up to 6 days with 25–50 μM genistein dissolved in 0.1 M Na_2CO_3, but did not see any morphological signs of apoptosis although cell proliferation was apparently inhibited. Therefore, other factors such as differences in cell culture medium, source of purified genistein, and primary antibodies to detect *Bax* and *Bcl-2* may explain the differences in results. Furthermore, it is possible that there could be MDA-MB-231 cells with dissimilar phenotypes as a result of the cells being maintained under different conditions in different laboratories, as suggested recently for MCF-7 cells [Gooch and Yee, 1999].

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