Bcl-2 overexpression sensitizes MCF-7 cells to genistein by multiple mechanisms

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Abstract. Genistein is a soy isoflavone with anti-tumor properties. Genistein-induced apoptosis involves Bcl-2 down-regulation. However, overexpression of Bcl-2 in breast cancer has been associated with better prognosis and response to hormonal therapy. To examine genistein's effect on breast cancer cells with different Bcl-2 levels, we established control (MCF-7/PV) and Bcl-2 overexpressing MCF-7 (MCF-7/Bcl-2) cell lines and characterized genistein regulated apoptosis and cell cycle progression in these cells. Our results demonstrate that overexpression of Bcl-2 rendered MCF-7 cells more sensitive, rather than resistant, to genistein. We found that genistein induces enhanced cytochrome c release and mito-chondrial membrane depolarization in MCF-7/Bcl-2 cells, as compared to control. We also found that genistein increases Bcl-2 levels and Bcl-2/Bax ratio in the mitochondrial fractions of MCF-7/Bcl-2 cells, suggesting that disturbed Bcl-2/Bax distribution may cause cytochrome c release and apoptosis in these cells. Cell cycle analysis indicated that genistein induces G0/G1 arrest in MCF-7/PV cells but increases in G2/M arrest in MCF-7/Bcl-2 cells. This was accompanied by modified responses of several cell cycle regulators, such as p21 and cyclin B1. Taken together, our results indicate that genistein-Bcl-2 interaction switches Bcl-2 from an anti-apoptotic protein into a proapoptotic protein, which involves disturbed Bcl-2/Bax distribution in mitochondria, increased cytochrome c release and modified cell cycle regulation.

Introduction

High intake of soy products has been associated with low incidence of breast cancers in Asian countries (1,2). Many soy-mediated anti-tumor properties have been attributed to

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genistein, a major soy isoflavone (3). Results from both *in vitro* and animal studies indicate that genistein may inhibit tumor cell growth by multiple mechanisms (3-5). As a typical phyto-estrogen, genistein binds to estrogen receptors (ERs) and modulates hormonal signaling of breast epithelial and cancer cells (6-8). For ER positive breast cancer cells, genistein inhibits cancer cells at higher concentrations, but stimulates cell growth at lower concentrations (9,10). Genistein is also known as a potent inhibitor of tyrosine kinases, such as EGFR (11-13), and a topoisomerase inhibitor (14). In addition, it was reported that genistein can activate the JNK pathway and inhibit NF-κB activity (15,16). As a result, integrated signaling from these pathways induces cell cycle arrest and, in particular, apoptosis, both of which play important roles in genistein mediated anti-tumor activities (3,17,18).

Genistein-induced apoptosis has been examined in a variety of cancer cell lines, including breast, prostate, pancreatic and lung cancers (3). Most reports indicate that genistein mediated apoptosis was accompanied by modulation of many p53 target genes, such as p21 and Bax, and the activation of the mitochondrial pathway (18,19). However, genistein-induced apoptosis could be p53 independent, because many cell lines that have p53 mutation were sensitive to genistein (7,18). On the basis of Bax upregulation and Bcl-2 downregulation in genistein treated cells, it was proposed that increase of Bax/Bcl-2 ratio is a key factor that initiates apoptotic cascade through the mitochondrial pathway (18,20). Nevertheless, the involvement of Bcl-2 in genistein-induced apoptosis was merely based on the association between growth inhibition and the Bcl-2 downregulation. The role of Bcl-2 in genistein-induced apoptosis remains to be defined.

Bcl-2 is the founding member of the Bcl-2 family, which consists of both anti-apoptotic and pro-apoptotic proteins, such as pro-apoptotic Bax, Bak and Bid, and anti-apoptotic Bcl-2, Bcl-xL and Bcl-W (21,22). The proapoptotic proteins Bax and Bak induce cell death by modulating mitochondrial membrane permeability, which leads to the release of cyto-chrome c and the activation of caspase cascade (23). By forming heterodimers with pro-apoptotic family members, Bcl-2 inhibits Bax/Bak activation and prevents the release of cytochrome c, resulting in cell survival (24). Overexpression of Bcl-2 is generally associated with tumor development,

progression and drug resistance, especially in leukemia (25,26). Although Bcl-2 overexpression is implicated in cell survival in most cases, increasing reports indicate that Bcl-2 over-expression is associated with a better prognosis in many solid tumors (27,28). In particular, Bcl-2 is overexpressed in about 40-70% of breast cancers. Overexpression of Bcl-2 in these tumors was associated with better overall outcomes and responses to hormonal therapy (29).

Previous studies have shown that genistein downregulates Bcl-2 in different cells (3). Little is known about the effect of genistein on cells with different Bcl-2 levels. Given that breast cancers with different Bcl-2 status have distinct prognostic significance and therapeutic responses (30), we questioned whether cells with different Bcl-2 levels respond to genistein differently. Based on control and Bcl-2 overexpressing MCF-7 cell line models, we found that genistein induces enhanced cell death and growth inhibition in MCF-7 cells overexpressing high levels of Bcl-2. Our results suggest that genistein interacts with Bcl-2 at multiple levels, and that breast cancers with high levels of Bcl-2 might benefit more from genistein treatment.

Materials and methods

Reagents. Genistein, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), DMEM/F12 medium and other chemicals were purchased from Sigma (St. Louis, MO). Antibodies against Bcl-2, p21, p27, cyclin D1, p53, Poly ADP-ribose polymerase (PARP) and Actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against cyclin B1 was from Rockland Immunochemicals (Gilbertsville, PA). Horseradish peroxidase (HRP)-labeled secondary antibodies were purchased from Amersham/GE Health Care (Arlington Height, IL). pCR-Bcl-2 plasmid was a generous gift from Dr John Reed (Burnham Institute, CA).

Cell culture and transfection. MCF-7 breast cancer cell line was purchased from American Type Culture Collection (ATCC). The cells were cultured in DMEM/F12 medium containing 10% fetal bovine serum (FBS). For drug treatment, $1.5x10^6$ cells were inoculated into a 60 mm dish 24 h before treatment. The cells were then treated with genistein at indicated concentrations for various durations. The control cells were treated with 0.1% DMSO. For transfection, $3x10^5$ cells were inoculated into a 35-mm dish 24 h before transfection. Transfection was performed using FuGENE 6 (Roche, IN) according to the manufacturer's protocol. Bcl-2 overexpressing MCF-7/Bcl-2 cell line was established by transfecting MCF-7 cells with pCR-Bcl-2 plasmid encoding human Bcl-2 cDNA, followed by G418 selection. G418 resistant clones were pooled for further characterization. The control cells were MCF-7 cells transfected on an empty pBabe/puro vector (MCF-7/pv) (31). MCF-7/C3/Bcl-2 cell line was established by transfection of MCF-7/C3 (31) cells with pCR-Bcl-2 followed by G418 selection.

MTT assay. For MTT assay, the cells were inoculated into 96-well plates at $1x10^3$ cells/well 24 h before the treatment. The cells were treated with increasing concentrations of genistein (0.023-50 μM) for 6 days. After removal of the

medium, 50 μl of MTT solution (500 μg/ml) was added to each well followed by 4-h incubation. MTT solution was replaced by DMSO to dissolve blue formazan crystals, and absorbance was measured at 562 nm using a microplate reader. Survival fractions were calculated based on MTT incorporation in the treated cells over the control groups of each cell line. Eight parallel samples were tested. Statistical difference was analyzed using Student's t-test.

Clonogenic assay. Six hundred cells were seeded into 60-mm dishes for 24 h before treatment. The cells were then treated with different doses of genistein (0, 2.5, 5, 10 and 20 μM) for 12 days in culture. Cell colonies were stained with 0.5% crystal violet in 25% methanol for 10 min followed by three rinses with water. The number of survival colonies was counted, and pictures were taken using UPV II imaging system. The experiments were performed in triplicate. Statistical difference was analyzed using Student's t-test.

Western blotting. The cells were lysed with lysis buffer containing 50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40 and protease inhibitors. Protein concentration was determined using BCA assay kit (Pierce Biotechnology Inc. IL). Fifty μg of protein lysate was loaded onto each lane of a gel. Proteins were separated with 12% or 15% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with a specific primary antibody at a dilution of 1:300 to 2000, followed by washing and probing with a corresponding secondary antibody. The specific protein band was visualized by autoradiography using an ECL kit (GE Health Care).

Cytochrome c release assay. The cytosolic extract for cyto-chrome *c* release experiments was prepared according to previously published protocol (32). In brief, treated cells were washed with PBS and resuspended in sucrose containing buffer. After 30-min incubation, the cells were disrupted by 30 strokes with a glass Dounce (Type B). The large debris in the cell homogenates was cleaned by centrifugation at 880 g for 10 min. Mitochondria in the supernatant were separated by further centrifugation at 22,000 g for 15 min. The resulting supernatants were saved as cytosolic extract. Cytochrome *c* in this extract was detected using Western blotting described above.

Flow cytometry for cell cycle analysis. MCF-7/PV and MCF-7/Bcl-2 cells were treated with different concentrations of genistein for 48 h. The cells were then fixed with 80% ethanol. After being washed three times with PBS containing 0.1% Triton X-100 and treated with RNase A (1 mg/ml) for 30 min, the cells were stained with 50 μg/ml propidium iodide. Cell analysis was performed using a FACSCalibur flow cytometer (Becton-Dickinson). The fraction of the total cell population present in the G1, S phase, G2-M phase was obtained from DNA histograms by mathematical modeling using MultiFit software.

Analysis of mitochondrial depolarization. Mitochondrial depolarization was examined by JC-1 staining and flow cytometry. This assay is based on the principle that fluorescence of JC-1 changes with mitochondrial membrane potential. Red
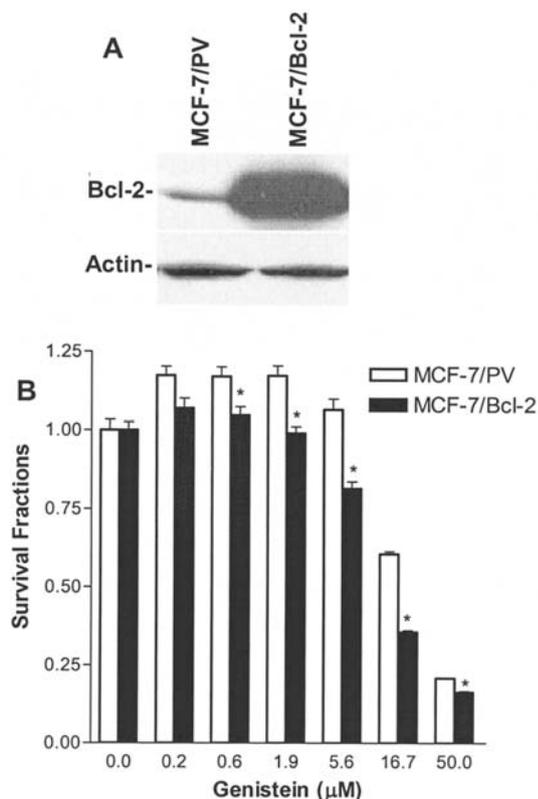
Figure 1. Bcl-2 overexpression in MCF-7 cells sensitizes the cells to genistein. A, Establishment of control and Bcl-2 overexpressing MCF-7 cells. MCF-7 cells were transfected with pCR/Bcl-2 or control vector (PV) followed by G418 selection. G418 resistant clones were pooled for further studies. Bcl-2 levels in MCF-7/PV and MCF-7/Bcl-2 cells were detected by Western blotting. B, Effects of genistein on MCF-7/PV and MCF-7/Bcl-2 cell proliferation. MCF-7/PV and MCF-7/Bcl-2 cells were treated with genistein at indicated concentrations for 6 days, followed by MTT assay. Survival fractions of the treated cells, based on 8 parallel samples, were statistically analyzed. *P<0.01.

fluorescence indicates intact mitochondria. As mitochondrial depolarization occurs the cells gain green fluorescence and lose red fluorescence in particular. In these experiments, genistein treated cells were trypsinized, followed by washing with PBS. The cells were then stained with 1 μM JC-1 (Molecular Probes, Eugene, OR) in serum free DMEM/F12 medium at 37˚C for 20 min, followed by washing with color free DMEM/F12. For flow cytometry analysis, detectors FL-1 and FL-2 were set to measure green and red fluorescence respectively. The percentage of cells in each quadrant was calculated using the WinMDI 2.8 software. Cells in the lower right quadrant represent the cells with mitochondrial depolarization.

Results

Bcl-2 overexpression sensitizes MCF-7 cells to genistein. In order to determine the effect of Bcl-2 overexpression on genistein-induced growth inhibition, we first established control (MCF-7/PV) and Bcl-2 overexpressing MCF-7 (MCF-7/Bcl-2) cell lines as detailed in Materials and methods. As shown in Fig. 1A, MCF-7/Bcl-2 cells express significantly higher levels of Bcl-2 than the control cells. We then examined the responsiveness of the two isogenic cell lines to genistein using MTT assay. To our surprise, cells overexpressing
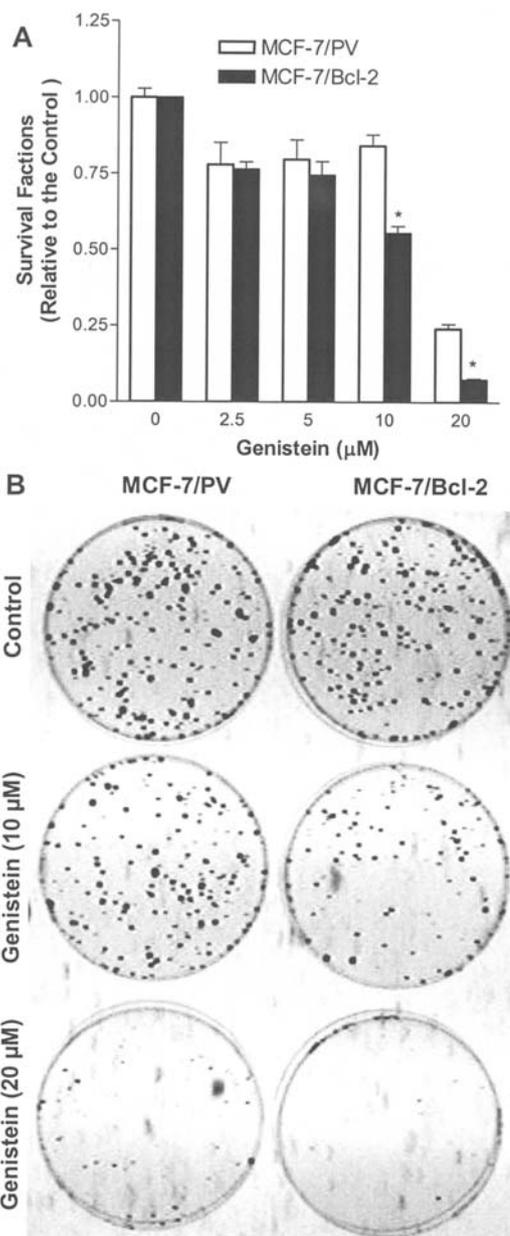

Figure 2. Enhanced growth inhibition in genistein treated MCF-7/Bcl-2 cells determined by clonogenic assay. MCF-7/PV and MCF-7/Bcl-2 cells at 600 cells/60-mm dish were treated with genistein (control/DMSO, 10 or 20 μM) for 12 days, followed by crystal violet staining. The number of survival colonies on each dish was counted, and the pictures were taken using UPV II Imaging system. The experiments were performed in triplicate. *p<0.01.

anti-apoptotic Bcl-2 were more sensitive to genistein than the control cells in a dose-dependent manner (Fig. 1B). Genistein treated MCF-7/PV cells displayed a bi-phasic response, i.e., enhanced proliferation at lower doses (<5.6 μM) but inhibited growth at higher concentration, which is consistent with previous reports (33). In contrast, the survival fractions of genistein treated MCF-7/Bcl-2 cells were consistently lower as compared to that of MCF-7/PV cells under the same conditions.

To confirm that Bcl-2 overexpressing cells are more sensitive to genistein, we performed clonogenic assays, in which control and MCF-7/Bcl-2 cells were treated with 0, 2.5, 5, 10 and 20 μM of genistein for 12 days. As shown in Fig. 2, the survival fractions of all genistein treated MCF-7/
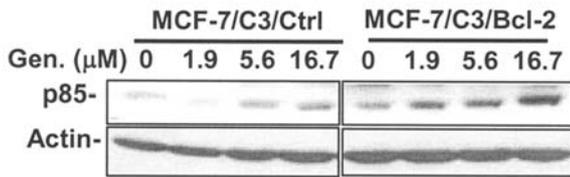
Figure 3. PARP cleavage in caspase-3 reconstituted MCF-7 cells (MCF-7/C3) with/without Bcl-2 overexpression. MCF-7/C3 control (MCF-7/C3/Ctrl) and MCF-7/C3/Bcl-2 cells were treated with genistein (Gen.) at indicated concentrations for 48 h. Production of p85, a major subunit of cleaved PARP, was detected using Western blotting.
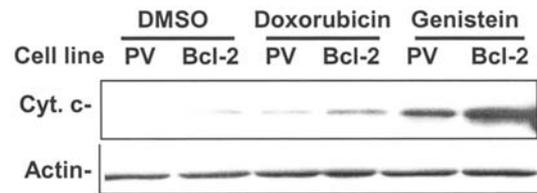

Figure 4. Enhanced cytochrome *c* release from the mitochondria of genistein treated MCF-7/Bcl-2 cells. MCF-7/PV (PV) and MCF-7/Bcl-2 (Bcl-2) cells were treated with 0.1% DMSO, 1 μM doxorubicin or 50 μM of genistein for 48 h. Cytosolic fractions were extracted from the treated cells using dunce homogenizer. Cytochrome *c* (Cyt. *c*) in the cytosolic fractions was detected using Western blotting.

Bcl-2 groups were lower than MCF-7/pv cells. The most significant differences between the two cells lines were observed in 10 and 20 μM groups. These results demonstrate that MCF-7 cells overexpressing Bcl-2 were indeed more sensitive to genistein than low Bcl-2 expressing cells.

PARP cleavage in genistein treated MCF-7C3//Bcl-2 cells. Caspase-mediated cleavage of PARP is a common marker of apoptosis. We tried to use PARP cleavage to indicate the enhanced apoptosis in genistein treated MCF-7/Bcl-2 cells. However, due to caspase-3 deficiency in MCF-7 cells (31), PARP cleavage was not sensitive in these cells (data not shown). We then established MCF-7/C3/Bcl-2 cell line [MCF-7/C3 cells are MCF-7 cells reconstituted with caspase-3 (31)] and examined PARP cleavage in genistein treated MCF-7/C3/control and MCF-7/C3/Bcl-2 cells. As shown in Fig. 3, genistein treatment results in increased levels of p85, a major subunit of cleaved PARP, in MCF-7/C3/Bcl-2 cells as compared to MCF-7/C3/control cells. This result suggests that enhanced activation of caspase cascade is involved in Bcl-2 overexpression mediated sensitization of MCF-7 cells to genistein.

Genistein induces enhanced cytochrome c release and mitochondrial membrane depolarization in Bcl-2 overexpressing MCF-7 cells. Since the immediate functional target of Bcl-2 is mitochondrial membrane permeability, we examined the effect of genistein on cytochrome *c* release from the mitochondria of control and MCF-7/Bcl-2 cells. Cytosolic fraction was extracted from each of the cell lines treated with DMSO, 50 μM of genistein or 1 μM of doxorubicin (serving as a control of DNA damaging agent) for 48 h. Under the given conditions, cytochrome *c* in the cytosolic fraction of DMSO treated MCF-7/PV cells was not detectable (Fig. 4). A weak band of cytochrome *c*, however, was detected in DMSO treated MCF-7/Bcl-2 cells. After doxorubicin or genistein treatment, cytochrome *c* levels in the cytosolic fractions of each cell line were increased as compared to their corresponding control. More importantly, the increase in genistein treated MCF-7/Bcl-2 cells was the most significant. These results suggest that cytochrome *c* release was more susceptible in MCF-7/Bcl-2 cells, which may lead to enhanced apoptosis in these cells.

To further characterize the effect of genistein on mitochondrial membrane permeability, we examined mitochondrial membrane depolarization using JC-1 staining. In cells with intact mitochondria, JC-1 accumulates as aggregates in the
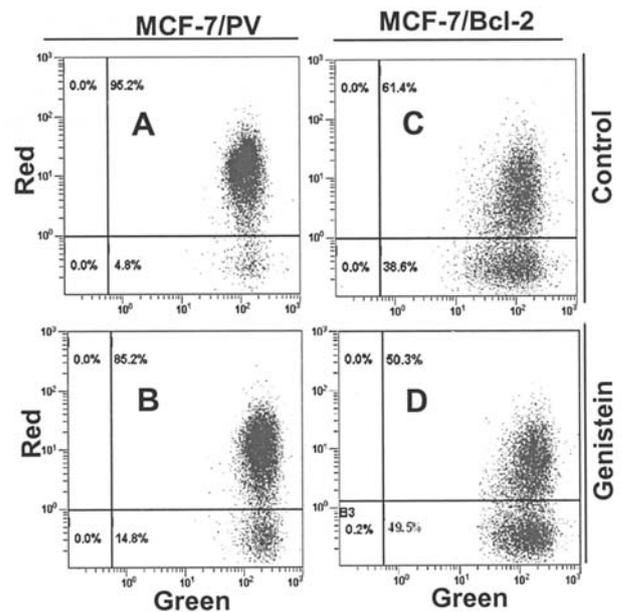

Figure 5. Mitochondrial depolarization in control and genistein treated MCF-7/PV and MCF-7/Bcl-2 cells. Cells of each line were treated with 0.1% DMSO or 25 μM of genistein for 24 h. The cells were then trypsinized and stained with 1 μM of JC-1 for 20 min. The red and green fluorescence of the cells stained with JC-1 was analyzed using flow cytometry as described in Materials and methods. Cells in the lower-right quadrant are the cells with mitochondrial depolarization.

mitochondria, resulting in red fluorescence. Depolarization of mitochondria in JC-1 stained cells results in a decrease in red fluorescence and an increase in its green fluorescence (34). Based on flow cytometry analysis (Fig. 5), we found that genistein only induced a moderate mitochondrial depolarization (the cells in the lower-right quadrant) in MCF-7/PV cells (Fig. 5B). In contrast, MCF-7/Bcl-2 cells displayed a higher background in mitochondrial depolarization (Fig. 5C). In particular, the percentage of genistein treated MCF-7/Bcl-2 cells with mitochondrial depolarization was much higher than MCF-7/PV cells under the same conditions. These data, while showing that Bcl-2 overexpression alone may cause some spontaneous depolarization, further support the above finding that genistein induces enhanced mitochondrial membrane permeability changes and cytochrome *c* release in the cells expressing high levels of Bcl-2.

Genistein modulates the distribution of Bcl-2 and Bax in the mitochondrial fractions of MCF-7/PV and MCF-7/Bcl-2 cells.
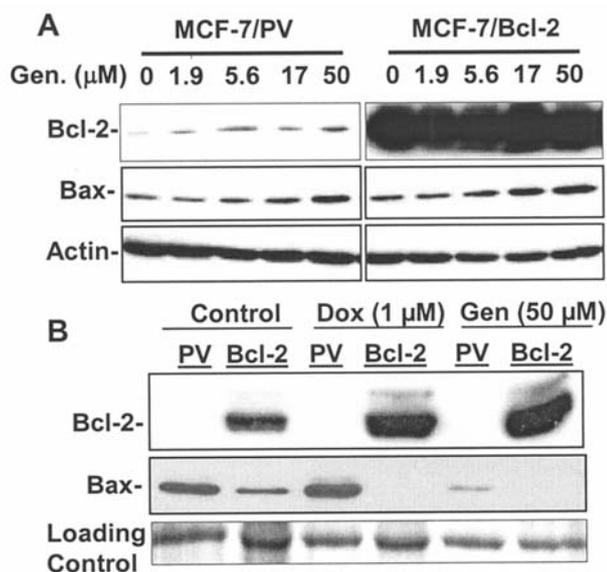
Figure 6. Bcl-2 and Bax levels in genistein treated MCF-7/pv (PV) and MCF-7/Bcl-2 (Bcl-2) cells. A, Bcl-2 and Bax levels in whole cell lysate. MCF-7/PV cells and MCF-7/Bcl-2 cells were treated with genistein at indicated concentrations for 48 h. Protein levels of Bax and Bcl-2 in the whole cell lysate were detected using Western blotting. B, Bcl-2 and Bax levels in the mitochondrial fraction. MCF-7/PV cells and MCF-7/Bcl-2 cells were treated with 0.1% DMSO (Control), 1 μM of doxorubicin (Dox) or 50 μM of genistein (Gen) for 48 h. Mitochondrial fractions were prepared as described in Materials and methods. Bax and Bcl-2 protein levels were detected using Western blotting. After probing with Bcl-2 and Bax, the membrane was stained with 0.1% amido black 10B. Signal of a 60-kDa non-specific band was used as the loading control.

Interaction between Bcl-2 and Bax is critical in the regulation of cytochrome *c* release. Upregulation of Bax and down-regulation of Bcl-2, or increased Bax/Bcl-2 ratio, has been observed in many studies of genistein-induced apoptosis (3). We therefore examined Bcl-2 and Bax levels in the whole cell lysates of MCF-7/PV and MCF-7/Bcl-2 cells treated with genistein. In MCF-7/PV cells, genistein modestly induced the expression of endogenous Bcl-2 at concentrations ranging from 1.9 to 50 μM (Fig. 6A). Bcl-2 levels in genistein treated MCF-7/Bcl-2 cells, however, were consistently high due to the expression of exogenous Bcl-2. Furthermore, genistein-induced Bax expression in both MCF-7/PV and MCF-/Bcl-2 cells, whereas more in the latter. In contrast to the increased Bax/Bcl-2 ratio in genistein treated MCF-7/PV cells, the ratio in MCF-7/Bcl-2 cells was consistently lower. These results suggest that increased Bax/Bcl-2 ratio in control cells might be a proapoptotic factor in the control cells, but not in MCF-7/Bcl-2 cells. It suggests that if Bax/Bcl-2 ratio exceeds a certain threshold, excessive Bcl-2 may promote cytochrome *c* release. In addition, since it was reported that Bcl-2 could be cleaved by caspases and turned into a Bax-like proapoptotic molecule (35), we particularly examined whether genistein induced Bcl-2 cleavage in these cells. We found that Bcl-2 cleavage was not detected in either cell line treated with genistein (data not shown).

Since distribution/translocation of Bcl-2 family members is a factor affecting their function (36), we then analyzed Bcl-2 and Bax distribution in the mitochondrial fraction. Under the given conditions, Bcl-2 was only detected in MCF-7/Bcl-2 cells (Fig. 6B). In contrast to no fluctuation in Bcl-2 levels in the whole cell lysate of MCF-7/Bcl-2 cells, a notable difference is that Bcl-2 levels in the mitochondrial fractions of genistein treated MCF-7/Bcl-2 cells increased significantly as compared to DMSO treated cells. Treatment with 1 μM of doxorubicin also induced moderate increase of Bcl-2 in the mitochondrial fractions. Increased Bcl-2 levels in mitochondrial fraction, but not in the whole cell lyste of MCF-7/Bcl-2 cells, suggest that genistein may induce Bcl-2 translocation to the mitochondria of these cells. Interestingly, Bax levels in mitochondrial fraction were inversely correlated with Bcl-2 levels. In DMSO treated control cells, Bax levels were lower in MCF-7/Bcl-2 cells as compared to MCF-7/PV cells. Doxorubicin increased Bax levels in MCF-7/PV mito-chondria but not in MCF-7/Bcl-2 cells. In response to genistein treatment, Bax levels were decreased in MCF-7/PV cells but not detectable in MCF-7/Bcl-2 cells. These results suggest that genistein induced excessive accumulation of Bcl-2 in the mitochondria of MCF-7/Bcl-2 cells, possibly due to translocation, and affected the anchorage of Bax protein in mitochondria. Disturbed Bcl-2/Bax distribution in mito-chondria may lead to increased cytochrome *c* release and consequent enhanced-apoptosis in these cells.

Genistein induces G2/M arrest in Bcl-2 overexpressing MCF-7 cells. Cell cycle arrest is another phenotype commonly asso-ciated with genistein-induced growth inhibition. To test whether Bcl-2 overexpression modulates genistein-induced cell cycle arrest, we treated MCF-7/PV and MCF-7/Bcl-2 cells with 50 μM of genistein for 24 and 48 h (Fig. 7). We found that 48-h treatment induced greater modification of cell cycle progression, although the patterns of cell cycle distribution of the same cell line at different time points were similar. As shown in Fig. 7, genistein induced cell growth arrest in both MCF-7/PV and MCF-7/Bcl-2 cell lines. However, in contrast to MCF-7/PV cells, which were mainly arrested in G0/G1 phases (Fig. 7B and F), genistein induced a significant increase of G2/M arrest in MCF-7/Bcl-2 cells (Fig. 7D and H). The results clearly indicate that genistein/ Bcl-2 interactions resulted in modified cell cycle arrest patterns, suggesting that alteration in cell cycle regulation may also contribute to the enhanced growth inhibition in genistein treated MCF-7/Bcl-2 cells.

Effects of genistein on cell cycle regulators in control and Bcl-2 overexpressing MCF-7 cells. With distinctive changes in growth inhibition and cell cycle arrest in MCF-7/Bcl-2 cells, as compared to the control cells, we examined the expression of several key cell cycle regulators, including p53, p21, p27, cdc-2 and cyclin B1. In general, genistein upregulated p53 and p21, and downregulated cdc-2 and cyclin B1 in both cell lines (Fig. 8), which is consistent with previous reports (7,37-39). p27 expression was not significantly affected by genistein. Comparison between the two cell lines reveals some differential effects. It appears that overall p53 levels in MCF-7/Bcl-2 cells were lower than MCF-7/PV cells. Nevertheless, upregulation of p21 in MCF-7/Bcl-2 cells was more evident than in the control cells, suggesting more factors might be involved in genistein-mediated upregulation of p21. In addition, cyclin B1 levels were also lower in MCF-7/
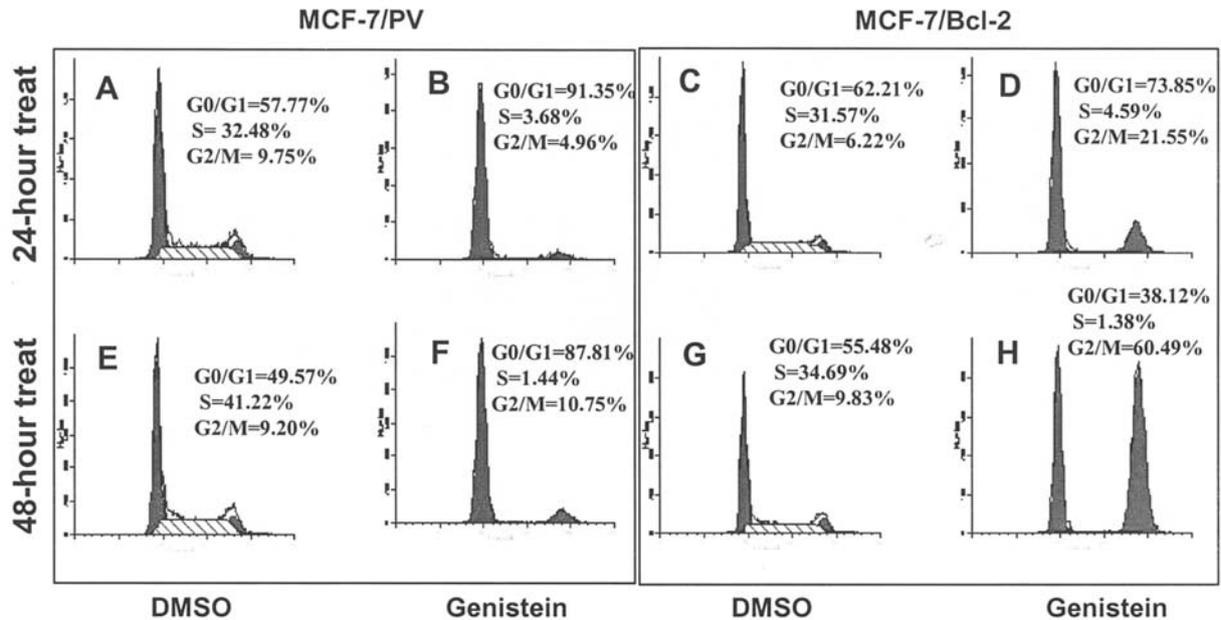
Figure 7. Overexpression of Bcl-2 modifies cell cycle arrest pattern in genistein treated cells. MCF-7/PV and MCF-7/Bcl-2 cells were treated with 0.1% DMSO or 50 μM of genistein for 24 or 48 h. Treated cells were then fixed with 80% ethanol, followed by RNase A treatment and propidium iodine staining. DNA contents were measured using a FACSCalibur. Cell cycle distribution was analyzed using MultiFit software. Representative data of three independent experiments are presented.
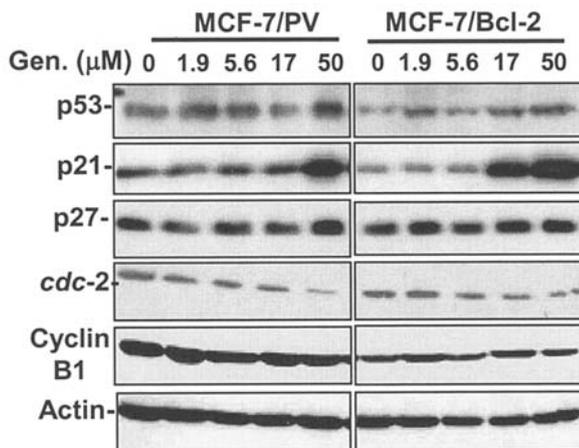

Figure 8. Effects of genistein on the expression of key cell cycle regulators in MCF-7/PV and MCF-7/Bcl-2 cells. Each cell line was treated with genistein at indicated concentrations for 48 h. Protein levels of p53, p21, p27, cdc-2, cyclin B1 and actin were detected using Western blotting.

Bcl-2 cells, as compared to control. Modified regulation of p21 and cyclin B1 by genistein in Bcl-2 overexpressing cells suggests that these factors may play a role in cell cycle arrest and sensitization resulted from Bcl-2-genistein interaction. Detailed mechanisms of genistein-mediated regulation of cell cycle progression in MCF-7/Bcl-2 cells will be followed in future studies.

Discussion

Numerous studies have demonstrated that cellular responses to genistein vary significantly in a cell line-dependent manner. Other than ER status, however, the cellular factors that

determine genistein responses are largely unknown. In this report, we focused on the effect of genistein on isogenic breast cancer cell lines with different Bcl-2 levels. We found that MCF-7 cells expressing high levels of Bcl-2 were more sensitive, rather than resistant, to genistein. This novel finding not only advances our understanding of genistein-mediated growth inhibition/apoptosis, but may also shed some light on the association between Bcl-2 overexpression and improved outcomes in breast cancers, a paradox observed in clinical studies.

Bcl-2 is a well known anti-apoptotic protein. It inhibits apoptosis and promotes cellular survival mainly by interfering with the translocation and subsequent oligomerization of Bax/Bak, which prevents the release of cytochrome *c* and other apoptotic factors from the mitochondria (21). Overexpression of Bcl-2 has been associated with tumor development and chemoresistance in some tumors (26). Despite these facts, increasing reports suggest that Bcl-2 may switch its role from an anti-apoptotic protein to a proapoptotic molecule under certain circumstances. One aspect of the switch was mediated by caspase cleavage. Bcl-2 can be cleaved by activated caspase-3, resulting in Bax-like proapoptotic fragment, which enhances apoptotic execution in a feedback manner (35). It was also reported that Bcl-2 overexpression in testicular germline tumor cells induces enhanced cell death by downregulating Bcl-xL (40). In a mechanistic study, it was reported that Bcl-2 is converted to a proapoptotic protein by interacting with nuclear receptor Nur77/TR3 (41). In addition, it was reported that transient high level expression of Bcl-2 induces cell death independent of Bax and Bak (42,43). Given the possible conversion of Bcl-2 into a pro-apoptotic protein, identification of the factors/conditions that may induce such a conversion would have significant implication in clinical oncology. Supported by the results

from both MTT and clonogenic assays, our data demonstrate that MCF-7 cells overexpressing high levels of Bcl-2 are more sensitive to genistein, suggesting that genistein is a factor that facilitates conversion of Bcl-2 into a proapoptotic protein.

To study the mechanisms of the enhanced apoptosis/growth inhibition in genistein treated MCF-7/Bcl-2 cells, we have examined a number of markers that are associated with either the effect of genistein or function of Bcl-2. Our results suggest a correlation between enhanced apoptosis and modified mitochondrial regulation in genistein treated MCF-7/Bcl-2 cells. It has been known that cytochrome *c*, along with AIF and Smac/Diablo, is released from the inter-membrane space of mitochondria into the cytosol through the mitochondrial permeability transition pore (PTP) (42,43). Loss of mitochondrial potential (membrane depolarization) induces PTP formation (44,45). Therefore, increased mitochondrial depolarization and cytochrome *c* release appear to be a major step leading to enhanced apoptosis in genistein treated MCF-7/Bcl-2 cells. However, since Bcl-2 is best known as an anti-apoptotic protein that prevents cytochrome *c* release, the intriguing question is how genistein induces enhanced PTP formation and cytochrome *c* release. Clearly, this cannot be explained by simple Bax/Bcl-2 ratio in those cells, because MCF-7/Bcl-2 cells express high levels of Bcl-2. On the contrary, our results suggest that when Bcl-2 levels reach a certain threshold and become dominant over Bax levels, more Bcl-2 may promote PTP formation. Given that Bcl-2 levels were increased in the mitochondrial fractions of the treated MCF-7/Bcl-2 cells, it is possible that genistein induces Bcl-2 translocation and enriches Bcl-2 loading on mitochondrial membrane, which induced more PTP formation and cytochrome *c* release. How genistein induces Bcl-2 translocation and how Bcl-2 induces PTP formation requires further study. In particular, how Bcl-2 levels are inversely correlated with Bax needs further investigation. Since MCF-7/Bcl-2 cells are sensitive to the induction of mitochondrial depolarization and cytochrome *c* release, our cell lines may be a useful model for these studies. In addition, although Bcl-2 could be converted into a proapoptotic protein by caspase-3 cleavage and interaction with Nur77, we have not detected any Bcl-2 cleavage or Nur77 upregulation in either cell lines treated with genistein (data not shown). Therefore, these factors are unlikely to be the major cause of enhanced growth inhibition in MCF-7/Bcl-2 cells. However, whether Nur77 translocation was associated with Bcl-2 translocation warrants further study.

Increased cell cycle arrest at G2/M phases in genistein treated MCF-7/Bcl-2 cells suggests that modulation of cell cycle progression may also contribute to enhanced growth inhibition in these cells. Previous studies indicate that either genistein or Bcl-2 alone could modulate cell cycle progression. Genistein was reported to arrest LNCaP prostate cancer cells at G1 phase (46) but arrest MCF-7 and HCG-27 cells at G2/M phases (17,47). For Bcl-2 overexpression induced arrest, most reports indicate a G0/G1 arrest (48,49). However, Bcl-2 may also induce G2/M arrest by physically interacting with and inhibiting Cdk-2 (50). In our own experiments, we found that, after genistein treatment, the percentage of MCF-7/Bcl-2 cells in G2/M phase significantly increased

(Fig. 7D and H). We also found that genistein induces differential responses of p21, Bax and cyclin B1 between the two cell lines. The specific role of each of the modified factors in genistein-Bcl-2 interaction induced G2/M arrest in MCF-7/Bcl-2 cells will be addressed in future studies.

Our results may be linked to the clinical data regarding the role of Bcl-2 overexpression in breast cancer. Although most *in vitro* studies demonstrate that Bcl-2 is an anti-apoptotic protein and Bcl-2 overexpression is associated with chemoresistance with certain types of tumors, including leukemia, neuroblastoma, and small cell lung cancer. However, a review of multiple publications including more than 1000 breast cancer patients suggests that Bcl-2 overexpression is generally associated with a favorable outcome (29). Breast cancers overexpressing Bcl-2 also predict a better response to hormonal therapy and some regimens of chemotherapy, as compared to Bcl-2 negative cells (51,52). Current understanding of Bcl-2 regulation remains inadequate to explain this paradoxical issue. Our results are similar to clinical observations. It appears that Bcl-2 overexpressing cancers would benefit not only from hormonal therapy, but also from soy/genistein treatment. Our cell line model may be a useful tool to study this clinically relevant problem. Since genistein shares both hormonal modulator properties with tamoxifen and DNA damaging function with chemotherapeutic agents, further study is required to ascertain which of these properties is more closely related to genistein-induced conversion of Bcl-2 into a proapoptotic protein. The results from the mechanistic studies on genistein-Bcl-2 interaction may be helpful in the explanation of Bcl-2 as a favorable biomarker in breast cancers.

Acknowledgements

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