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Epidemiological studies suggest that the phytochemicals, genistein and daidzein, are the anti-cancer agents found in Asian diets which contribute to a decreased breast cancer risk. In the current study, we investigated the molecular mechanism by which genistein inhibits cell growth and induces apoptosis. Our data show that genistein induces G2/M cell cycle arrest, dose-dependent cell growth inhibition, down-regulation of Bcl-2 and cyclin B, and up-regulation of $p21^{WAF-1}$ at low concentrations (15-30μM) in MCF-7, MDA-MB 231, MDA-MB 435 and erbB-2 transfected MDA-MB 435 clones of breast cancer cells. In contrast, the effect of genistein on MCF-10A normal breast epithelial cells was significantly different than breast cancer cells or MCF-10AneoT cells. The cell cycle arrest, cell growth inhibitory effect, and up-regulation of p21 were much more pronounced in MCF-10AneoT compared to MCF-10A cells, as previously reported. We also observed a down-regulation of cyclin B1 (CycB1) in both cell lines, similar to that found in breast cancer cells. In addition, we could not find any evidence of apoptosis in genistein treated MCF-10A and MCF-10AneoT cells compared to breast cancer cells where apoptosis was observed. The significance of these results will further be explored by additional studies in the future.

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TABLE OF CONTENTS

Introduction: **Page 5**

Body: **Page 5-11**

- **(i) Data on Breast Cancer Cell Lines** **Page 5-7**
- **(ii) Data on MCF-10A Cells** **Page 7-8**
- **(iii) Conclusions** **Page 8-10**
- **(iv) References** **Page 11**

INTRODUCTION:

The hypothesis to be tested in this proposal is to investigate whether soy metabolites selectively eliminate aberrant breast epithelial cells (tumor cells) by altering the expression of specific cell cycle regulatory genes which, in turn, causes cell cycle arrest and induces apoptosis.

Tumors often occur as a result of uncontrolled and de-regulated cell cycle caused by an imbalance of CKIs and CDKs. Current knowledge dictates that the de-regulation of many classes of genes are involved in tumorigenesis, such as oncoproteins, tumor suppressors, signaling pathways, growth factors or cytokines, apoptosis inducers and potentiators (1). Protectors and promoters of apoptosis interact with each other, and the outcome of this interaction, apoptosis or survival, depends on the ratio of the death promoter to the death suppressor (2). These apoptosis regulators are part of, and interact with, the cell cycle machinery. Therefore, the purpose of this study is to explore the mechanism of the soy metabolites, particularly genistein, in breast epithelial cells by examining any changes in the levels of cell cycle regulatory proteins induced or inhibited. Our proposal will also investigate whether the observed changes in specific gene expression are consistent with apoptosis in breast cancer cells. All results were planned to be generated from MCF-7 breast cancer cells, as well as from a series of non-transformed and non-tumorigenic (MCF-10A) and ras-transformed tumorigenic MCF-10AneoT breast epithelial cell lines (3-7), in addition to Bcl-2 transfected MCF-10A cells (8). **During the course of our investigation, we also included other breast cancer cell lines that were found to be significant for our investigation, and the results are included with this progress report.** The data generated from the work of this proposal should provide a precise molecular picture of the apoptotic mechanism(s) leading to breast cancer cell death, as well as the molecular mechanism of the soy metabolite, genistein. **Hence, the overall purpose of this study is to elucidate the molecular mechanism(s) by which the soy metabolite, genistein, exerts its biological effects on non-tumorigenic and tumorigenic breast epithelial cells. It is important to note that we focused our studies in a series of selected cell lines based on our findings to-date in this project as outlined in this progress report.**

BODY:

The following section describes the progress to-date of our project. These results are comprehensive and provide experimental methods, results and discussion in relation to the statement of work as proposed in the original application. However, it is important to note that certain breast cancer cell lines have been included for our study in addition to those originally proposed.

Data on breast cancer cell lines:

1. As previously reported in our last progress report, genistein was found to inhibit the proliferation of a human breast cancer cell line–MCF-7 in a dose dependent manner when exposed for four days in culture with different concentrations of genistein. Genistein concentrations of 5, 15, 25 and 50μM clearly demonstrated progressive growth inhibition, and the concentration of 25 to 50μM was found to be optimal when cells were exposed for 3 days in culture. Similar results were also reported in the literature (9, 10), however, our data suggest an ID_{50} of about 30μM is optimal for cellular effect. We, therefore, decided to use 30μM of genistein for subsequent experiments and the results of which have been reported previously.

Based on these earlier results, we decided to investigate the effect of genistein on another human breast cancer cell line, MDA-MB 231. During the course of these studies, we used TPA for certain experiments and the results were very novel and interesting. Hence based on the data generated from the treatment of breast cancer cells MDA-MB 231 with TPA in the context of apoptosis, a manuscript was prepared and subsequently published in the journal "Oncogene" (see appended reprint with this progress report, article#1). Furthermore, this cell line was also extensively used to measure the molecular effects of genistein and the results of those studies were included in another manuscript which has recently been published in the journal "Oncogene" (see appended reprint with this progress report, article #2). The results reported in the article #2 are also summarized below as such.

Abstract: Breast cancer is the most common cancer among American women, whereas Asian women, who consume a diet traditionally high in soy products, have a relatively low incidence. Genistein is a prominent isoflavonoid in soy products and has been proposed as the agent responsible for lowering the rate of breast cancer in Asian women. We investigated the effects of genistein on cell growth and apoptosis-related gene expression in breast cancer cells MDA-MB-231. We found up-regulation of Bax and $p21^{WAF1}$ expressions and down-regulation of Bcl-2 and p53 expressions in genistein-treated cells. Furthermore, DNA ladder formation, CPP32 activation, and PARP cleavage were observed after treatment with genistein, indicating apoptotic cell deaths. Flow cytometry with 7-amino actinomycin D staining showed that the number of apoptotic cells increased with longer treatment of genistein. From these results, we conclude that genistein inhibits the growth of MDA-MB-231 breast cancer cells, regulates the expression of apoptosis-related genes, and induces apoptosis through a p53-independent pathway. The up-regulation of Bax and $p21^{WAF1}$ may be the molecular mechanisms by which genistein induces apoptosis, however, further definitive studies are needed. These results suggest that genistein may be a potentially effective chemopreventive or therapeutic agent against breast cancer.

2. Subsequently, we were interested in investigating whether isogenic cell lines derived from MDA-MB-435 transfected with Her-2/neu to overexpress Her-2/neu (tyrosine kinase) may have any differential effect when exposed to genistein. This study was designed because genistein is a known inhibitor of tyrosine kinase. We initially planned to demonstrate whether cells that over express Her-2/neu may be more susceptible to the genistein -induced cell death. For this reason, we chose Her-2/neu, also known as *c-erbB-2*. The*c-erb*B-2 gene is a key molecule for cancer metastasis. The *c-erb*B-2 gene encodes a glycoprotein which is a growth factor receptor with intrinsic tyrosine kinase activity (11, 12). Overexpression of the *c-erb*B-2 gene has been found in approximately 20-30% of human breast cancers, and the patients whose tumors overexpress *c-erb*B-2 gene have a significantly lower survival rate (13, 14). High levels of expression of *c-erb*B-2 have been positively correlated with invasion and metastasis in breast, ovarian, lung and prostate cancers (15-19). Tan et al. (19) found that transfection of *c-erb*B-2 into breast cancer cells increased the ability of invasion and metastasis, and that an increase of matrix metalloproteinases (MMPs) were detected in *c-erb*B-2 transfected cells (19). MMPs have been found frequently among malignant tumor cells (20) and play an important role in tumor invasion and metastasis (21-23). MMP-2 and MMP-9 are Type IV collagenase/gelatinase and degrade the helic domains of Type IV collagen, which is a main component of the basement membrane. These MMPs are believed to be key molecules for cancer invasion and metastasis (24-26). Therefore, overexpression of *c-erb*B-2 is correlated with increases of MMP secretion and metastatic potential in breast cancer cells in experimental metastasis assays (19).

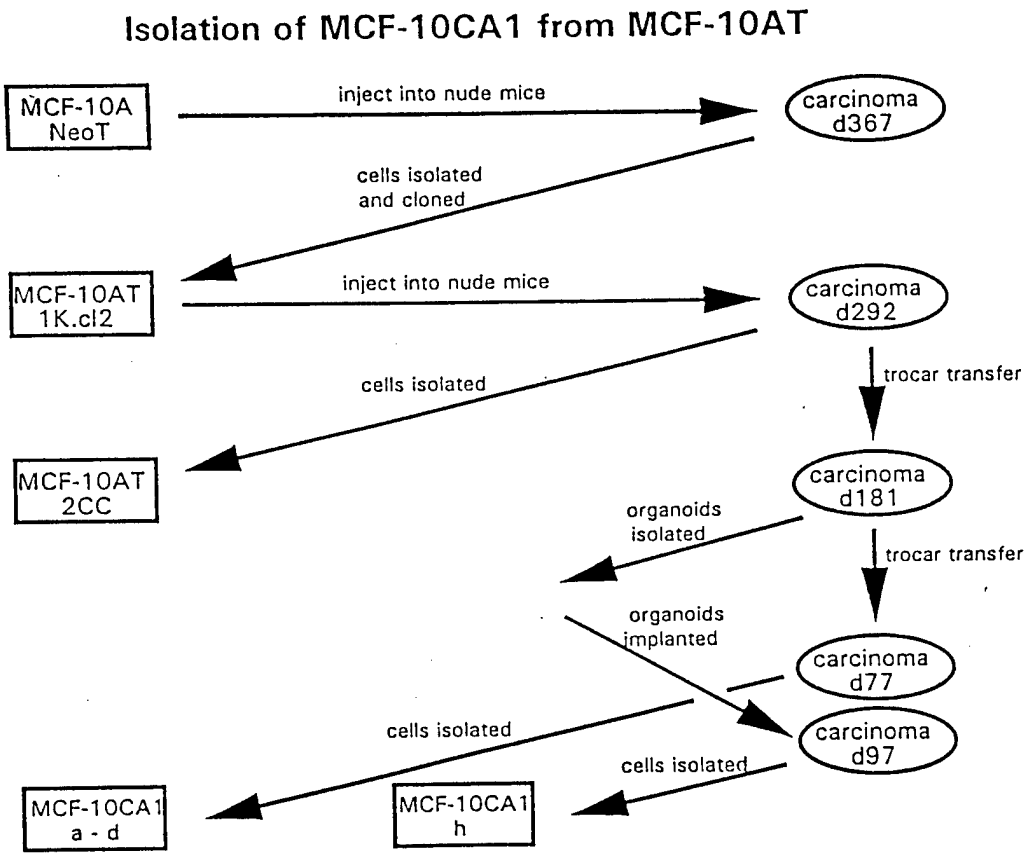
We have previously shown that genistein inhibits the growth of MDA-MB-231 breast cancer cells, regulates the expression of apoptosis-related genes, and induces apoptosis through a p53-independent pathway (Li et al. [27], see appended article). In the current study, we investigated these effects of genistein in the breast cancer cell line MDA-MB-435 and 435.eB cells that were established by transfecting *c-erb*B-2 cDNA into MDA-MB-435. We also investigated the effect of genistein on matrix metalloproteinase (MMP) secretion that was previously shown to be effected by *erb*B-2 transfection. Our data show that genistein inhibits the growth of MDA-MB-435 breast cancer cells, induces apoptosis, regulates the expression of genes, and may inhibit invasion and metastasis of breast cancer cells. These results are summarized below as such as an abstract, and the manuscript has been accepted for its publication in the "International Journal of Oncology" (see appended article, manuscript #3).

Abstract: Breast cancer is the most common cancer and second leading cause of cancer related deaths in women in the United States. Genistein is a protein tyrosine kinase inhibitor and prominent isoflavonoid in soy products and has been proposed as the agent responsible for lowering the rate of breast cancer in Asian women. We have previously showed that genistein inhibits the growth of MDA-MB-231 breast cancer cells, regulates the expression of apoptosis-related genes, and induces apoptosis through a p53-independent pathway. In this study, we investigated these effects of genistein in the breast cancer cell line MDA-MB-435 and 435.eB cells that were established by transfecting *c-erb*B-2 cDNA into MDA-MB-435. We also investigated the effect of genistein on matrix metalloproteinase (MMP) secretion previously shown to be effected by *erb*B-2 transfection. Genistein was found to inhibit MDA-MB-435 and 435.eB cell growth. Induction of apoptosis was also observed in these cell lines when treated with genistein, as measured by DNA laddering, poly(ADP-ribose) polymerase (PARP) cleavage, and flow cytometric analysis. We also found an up-regulation of Bax and $p21^{WAF1}$ expression and down-regulation of Bcl-2 and *c-erb*B-2 in genistein-treated cells. Gelatin zymography showed that genistein inhibits the secretion of MMP in the breast cancer cells. From these results, we conclude that genistein inhibits the growth of MDA-MB-435 breast cancer cells, induces apoptosis, regulates the expression of genes, and may inhibit invasion and metastasis of breast cancer cells. These findings suggest that genistein may be a potentially effective chemopreventive or therapeutic agent against breast cancer.

In summary, we have generated a substantial amount of data using various cell lines, which collectively demonstrate that genistein may be an universal agent for inhibiting breast cancer cell growth and that the induction of apoptotic cell death may be universal. These results are original and demonstrate for the first time the molecular mechanism by which genistein elicits its biological effects on human breast cancer cells. Furthermore, the results of these studies clearly show that the effect of genistein is neither dependent on the inhibitory activity of tyrosine kinase nor on the status of estrogen receptor. Hence, we are not investigating the effect of daidzein any further. All our subsequent experiments will be done with genistein alone to complete the project. Similar experiments are also ongoing to investigate the effect of genistein on a breast cancer cell model as demonstrated below.

(ii). ***Data on MCF-10A cell lines*:** As previously reported, in contrast to MCF-7 cells, the effect of genistein on MCF-10A (normal breast epithelial cells, see references, 3-7) was significantly different. Similarly, the effect of MCF-10A was also different than those observed with MDA-MB-231 and various isogenic cell lines derived from MDA-MB-435. The results of these experiments are ongoing, where we are investigating the effect of genistein in three MCF-

10A derived cell lines: MCF-10A (non-tumorigenic breast epithelial cells), Ras-transfected MCF-10AneoT derived MCF-10-TG3B (low tumorigenic) and a highly tumorigenic cell line MCF-10A-CA1a (see the chart below).



In summary, we have shown that genistein has no significant growth inhibitory activity at low concentration on normal breast epithelial cell line, MCF-10A, but has a significant effect on Ras–transformed MCF-10A cells (MCF-10AneoT and subsequent transplant generation, TG-3B). Thus, these model cell lines should provide critical data correlating the growth inhibitory effects of genistein with progression of normal epithelial cells to carcinoma and its association with the modulation in gene expression. These results should firmly establish the molecular mechanism of action of the soy metabolite, genistein, in the chemoprevention of breast cancer. **In addition, this model should also provide an unique *in vivo* model to test the chemopreventive role of dietary soy in breast cancer development and progression in future animal studies.**

Conclusions:

The data generated during the first two years of this project is very significant to the understanding of the molecular mechanism of action of genistein in breast epithelial cells. The summary and the conclusions of our results obtained during the first two years are detailed below as they relate to the specific objectives and the statement of work as proposed.

Objective: Specific Aim-1 was to demonstrate the effect of genistein and daidzein on non-tumorigenic and tumor cell lines derived from human breast tissues. Studies were planned to use MCF-10 and ras-transformed MCF 10AneoT human breast derived epithelial cell series and a series of breast tumor cell lines based on differential gene expression (i.e., estrogen receptor status, Her-2/neu and p53 status). The plan was to examine cell growth and cell cycle distribution by analyzing cell growth curves and cell cycle analysis by flow cytometry.

Progress: This part of our experimental plans has been fully completed and the data obtained from these experiments according to the statement of work has produced three original articles, of which two have been published and the third is in press (see appended articles). The conclusion of our investigation is that genistein appears to be rather selective under low concentration in causing the demise of cancer cells and that this data is very important in order to justify further chemoprevention studies using soy isoflavones. Our second conclusion is that genistein appears to be universally effective in breast cancer cells irrespective of their estrogen receptor status and the status of tyrosine kinase (erbB-2) overexpression or the mutational status of p53. Since tyrosine kinsae activity did not altered the effects of genistein in breast cancer cells, we are not investigating the effects of daidzen any further because the anti-proliferative and apototic activity of genistein is not dependent on the status of estrogen receptor or tyrosine kinase activity of erbB-2. Hence, we will complete our studies with genistein alone, but we will expand our molecular studies to elucidate the signaling pathways through which genistein elicits its biological activity in breast epithelial cells (see future plan section).

Objective: Specific Aim-2 was to understand the mechanism of action of genistein and daidzein, which leads to their anti-proliferative effect on human breast cells by:

(a) determining if apoptosis occurs in non-tumorigenic breast epithelial cells, as well as in breast cancer cells, after treatment with genistein and daidzein by performing the TUNEL assay and looking for evidence of DNA laddering.
(b) demonstrating the differential expression of proteins that are associated with cell cycle regulation and/or apoptosis by Western and Northern blot analysis.
(c) examining the growth inhibitory effects of genistein and daidzein together and their effects on gene expression as planned in (b).

Progress: The objectives specified under this specific aim as indicated under the statement of work has largely been completed, except the detail molecular mechanism (as part of specific aim 3), such as the signal transduction and in-depth analysis of how cell cycle and apoptosis related genes are altered in genistein treated cells. This part of the work will be completed during the third year. **As indicated earlier, we have also published two articles and one article is in press related to specific aim 1 and 2. Furthermore, the results demonstrating the differential effects of genistein in non-tumorigenic vs. tumorigenic cells derived from MCF-10A cell lines will be communicated to a journal for its publication during the third year of our investigation. Moreover, the results on signaling cascade will be interesting and will be published separately.**

Objective: Specific Aim-3 was to dissect the molecular mechanism of genistein and daidzein by comparing the effect of traditional tyrosine kinase inhibitors on cell cycle regulatory proteins and apoptosis.

Progress: Since we already demonstrated that the effect of genistein is not dependent on the status of tyrosine kinase of erbB-2, we will not investigate the effect of daidzein any further. However, the detailed molecular investigation as proposed under this specific aim will continue and will be completed during third year as proposed.

Collectively, we have accomplished Tasks 1 through 4 almost completely, except for some repeat experiments with MCF-10A derived cell lines as indicated under the statement of work. We will fully complete task-5 during the third year of the project.

Future Plan:

- Investigate why and how genistein show differential effects on non-tumorigenic and tumorigenic MCF-10A derived cells
- What is the signaling consequence of p21 and Bax up-regulation
- What effects genistein has on signaling molecules such as MAP kinase

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Induction of apoptosis in breast cancer cells by TPA

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Bcl-2, Bax and p53 gene products have been linked to programmed cell death pathways. p21^{WAF1} has been shown to mediate p53-induced cell cycle arrest and to inhibit cyclin-dependent kinase activity. We have analysed the expression of these genes and apoptosis induced by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) in several human breast cancer cell line. We found up-regulation of p21^{WAF1} and Bax expressions, however, the expressions of p53 and Bcl-2 genes remained unchanged in TPA-treated cells. Furthermore, DNA ladder formation and PARP cleavage were observed after treatment for 24 h, indicating apoptotic cell death. Flow cytometry with 7-amino actinomycin D staining showed that the number of apoptotic cells increased with longer treatment of TPA. From these results, we conclude that TPA is not only a tumor promoter, but also induces apoptosis in breast cancer cells. TPA-induced apoptosis appears to be mediated through a p53-independent pathway, and the up-regulation of p21^{WAF1} and Bax may be the molecular mechanisms by which TPA induces apoptosis.

Keywords: apoptosis; breast cancer; TPA

Introduction

Apoptosis (programmed cell death) is a physiological mode of cell death characterized by an intact cellular membrane with chromatin condensation and degradation into discrete DNA fragments of 180 to 200 base pairs (Chiarugi *et al.*, 1994; Huang *et al.*, 1997). The molecular mechanism implicated in programmed cell death has been partially elucidated and the induction of apoptosis is partly mediated intracellularly by several genes, such as p53, Bcl-2 and Bax (Chiarugi *et al.*, 1994). The p53 tumor suppressor gene is a cell cycle regulator, able to induce cell cycle arrest to allow DNA repair or apoptosis (Vogelstein and Kinzler, 1992). The molecular mechanisms underlying p53 action imply transactivation of p53 dependent genes such as p21^{WAF1} (El-Deiry *et al.*, 1993). P21^{WAF1} is not only activated by the p53 protein, but it also blocks the cell cycle at G1 phase much like p53 itself. In addition, an increased level of p21^{WAF1} is associated in cyclin-containing complexes with decreasing cyclin-dependent activity in damaged cells destined to apoptosis (El-Deiry *et al.*, 1993; Harper *et al.*, 1993). Although its mechanism of action is still unclear, Bcl-2 functions as a suppressor of apoptotic death triggered by a variety of signals (Sedlak *et al.*, 1995) and is negatively regulated by wild type p53. A family of genes with clear-cut homology to the Bcl-2 prototype has been identified, which exhibits a paradoxical behavior, acting as apoptosis protectors or inducers. This family of proteins includes Bax, Bcl-xl, Bcl-xs, etc (Sedlak *et al.*, 1995). Bcl-2 overexpression has been shown to inhibit apoptosis induced by a variety of stimuli, whereas, a predominance of Bax to Bcl-2 accelerates apoptosis upon apoptotic stimuli (Williams and Smith, 1993). Interestingly, Bax is also transactivated by p53 (Miyashita and Reed, 1995).

12-O-tetradecanoyl-phorbol-13-acetate (TPA) is thought to be a tumor promoter, which can induce Cox-2 expression. Overexpression of Cox-2 has been reported in about 90% of colon tumors and the premalignant colorectal adenomas (Kargman *et al.*, 1995; Sano *et al.*, 1995). A specific Cox-2 inhibitor was shown to effectively suppress the development of chemically induced aberrant crypt foci, a putative premalignant lesion (Reddy *et al.*, 1996). Paradoxically, TPA has also been shown to induce apoptosis in prostate cancer cells, suggesting that TPA is not only a tumor promoter, but it may serve as a potential anti-tumor agent in certain systems. Since breast cancer is the most commonly occurring tumor among American woman (Parker *et al.*, 1996), we sought to investigate the effects of TPA in breast cancer cells. Here we report for the first time that treatment of breast cancer cell lines having different differentiation, metastasis and p53 status with TPA, induced apoptosis with concomitant alterations in the apoptosis related gene expression.

Results

Effects of TPA on cell growth

Treatment of all five breast cancer cell lines for 24–72 h with 10 nM TPA resulted in inhibition of cell proliferation. The effects of TPA on the cell growth of MCF7, BT20, SKBR3, MDA-MB-231 and MDA-MB-453 cells are depicted in Figure 1. TPA was more anti-proliferative on SKBR3, MDA-MB-231 and MDA-MB-453 than on MCF7 and BT20 (MDA-MB-231 > SKBR3 > MDA-MB-453 > MCF7 > BT20). Induction of cell proliferation could be the result of cell cycle growth arrest, induction of apoptosis and/or the inhibition of growth. We, therefore, investigated whether TPA could induce apoptosis in breast cancer cells.

Induction of apoptosis

TPA-induced apoptosis was observed in all five cell lines as illustrated by DNA ladder shown in Figure 2.

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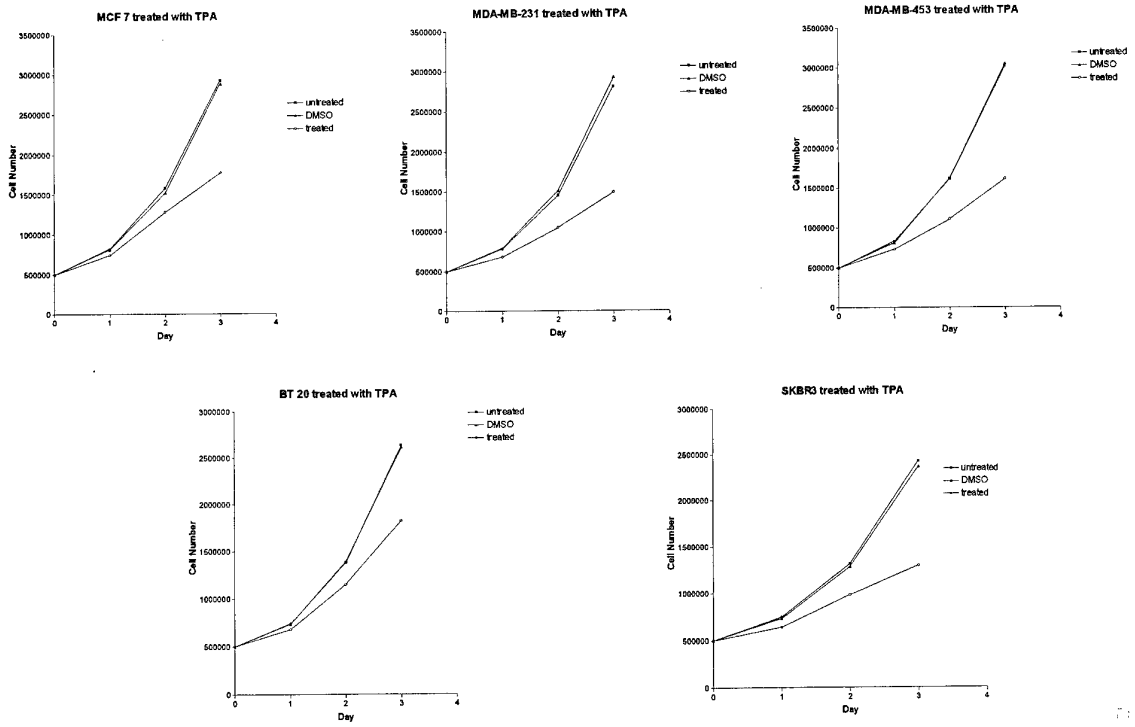

Figure 1 Cell growth inhibition by TPA

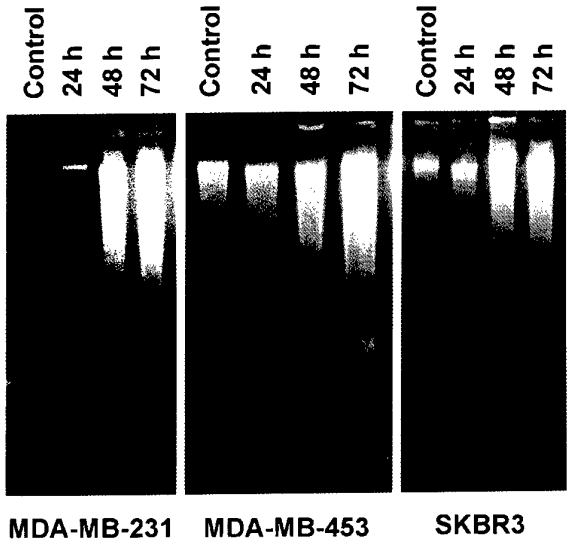

Figure 2 DNA ladder formation in breast cancer cells treated with TPA. (Control: Cells treated with DMSO for 48; 24, 48, 72 h: Cells treated with TPA for 24, 48, 72 h, respectively)

The induction of apoptosis was more pronounced in SKBR3, MDA-MB-231 and MDA-MB-453 cells at 48–72 h, and is directly correlated with the inhibition of cell growth. Western analysis of PARP cleavage also showed that the PARP protein was degraded, with the concomitant disappearance of full size (116 KD) molecule and accumulation of the 85 KD and further degraded product of about 52 KD, in the cells after 48 h of TPA treatment (Figure 3). This indicated the induction of apoptosis in the time point tested. Furthermore, the data of flow cytometry with 7-amino actinomycin D staining showed there were much more apoptotic cells after TPA treatment for 72 h (40.25%) and 96 h (56.57%), compared to control sample (6.42%) (Figure 4). Subsequently, we investigated the alterations in gene expression of p53 and p53 related genes involved in the apoptotic pathway.

Expression of $p21^{WAF1}$

The expression of $p21^{WAF1}$ in all five breast cancer cell lines was investigated in TPA-treated cells by Western and Northern blot analysis. The results of a typical experiment is shown in Figure 5, demonstrating that $p21^{WAF1}$ RNA was induced within 5 h of TPA treatment and down regulated to its basic level after 24 h. Western blot analysis, whereas, showed that the levels of $p21^{WAF1}$ protein reached maximum levels at 16 h and returned to control levels by 72 h of treatment (Figure 6). The induction of mRNA followed by its protein expression was directly correlated with the inhibition of cell growth.

Expression of Bcl-2 and Bax

The constitutive levels of Bcl-2 or Bax and the time course for the effect of TPA on Bcl-2 or Bax expression in the breast cancer cell lines were studied by Western and Northern blot analysis. The levels of Bcl-2 expression in the breast cancer lines were not significantly affected with the addition of 10 nM TPA to the cells. In contrast, the Bax RNA was induced after 24 h of TPA treatment (Figure 7) and the level of

Bax protein increased significantly in cells treated with TPA for 48 or 72 h (Figure 6). In order to obtain a quantitative value for the protein expression of Bax and Bcl-2, optical density measurement was conducted as described under Materials and methods. The ratios of Bax to Bcl-2 protein expression revealed that cells treated with TPA showed at least a 1.5-fold increase in Bax compared to Bcl-2 (Figure 8).

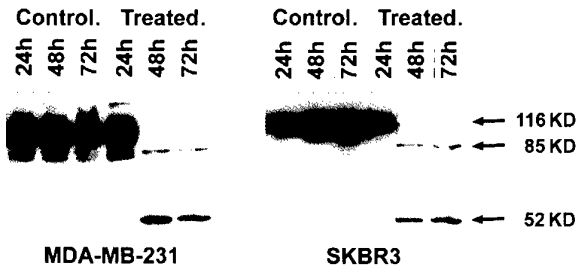

Figure 3 Western blot analysis of PARP cleavage. (Control: Cells treated with DMSO; Treated: Cells treated with TPA)

Expression of p53

The expression of p53 in the breast cancer cell lines with and without TPA treatment was tested with Northern and Western blot analysis. As showed in Figure 5 and 6, the levels of p53 RNA and protein in the breast cancer cell lines were not affected.

Discussion

Fragmentation of cellular DNA at the internucleosomal linker regions has been observed in cells undergoing apoptosis, which was induced by a variety of agents. This cleavage produces ladders of DNA fragments that are the sizes of integer multiples of a nucleosome length (180–200 bp) (Chiarugi *et al.*, 1994; Huang *et al.*, 1997; Fisher, 1994). Because of their characteristic patterns revealed by agarose gel electrophoresis, these nucleosomal DNA ladders are widely used as biochemical markers of apoptosis. The cleavage of poly(ADP-ribose) polymerase (PARP) has also been used as an early marker of chemotherapy-

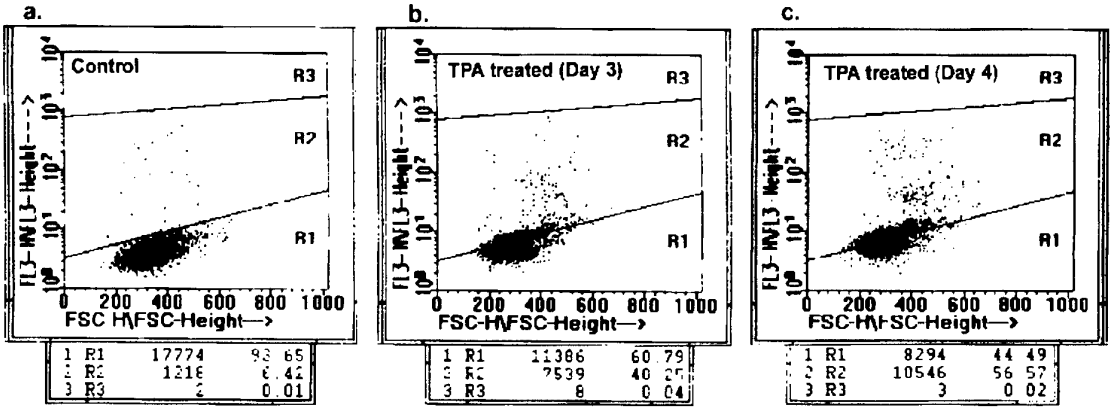

Figure 4 Scattergrams of 7AAD stained cells. (**a**) Control cells treated with DMSO for 96 h. (**b**) Cells treated with TPA for 72 h. (**c**) Cells treated with TPA for 96 h. (R1: live cells; R2: apoptotic cells; R3: dead cells)

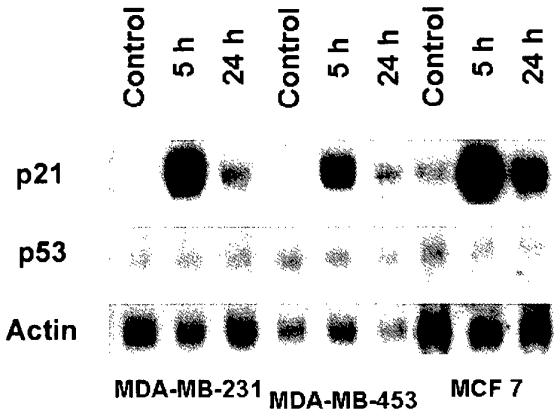

Figure 5 Northern blot analysis of $p21^{WAF1}$ and p53 in MCF7, MDA-MB-231 and MDA-MB-453. (Control: Cells treated with DMSO for 5; 5, 24 h: Cells treated with TPA for 5 and 24 h, respectively)

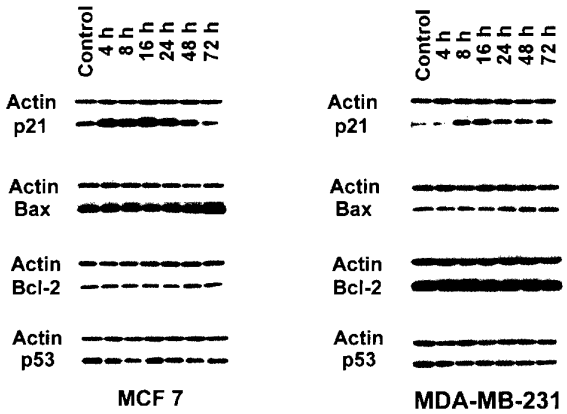

Figure 6 Western blot analysis of $p21^{WAF1}$, p53, Bax and Bcl-2 in MCF7 and MDA-MB-231. (Control: Cells treated with DMSO for 48; 4 , 8, 16, 24, 48, 72 h: Cells treated with TPA for 4, 8, 16, 24, 48, and 72 h, respectively)

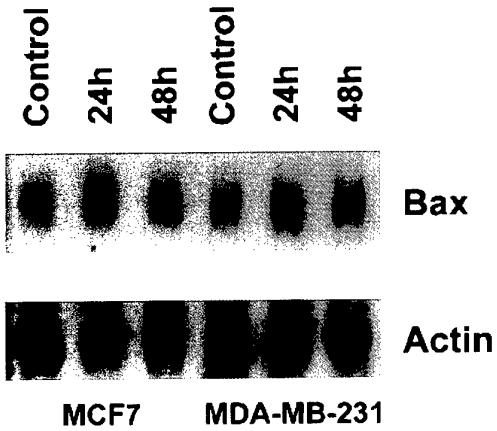

Figure 7 Northern blot analysis of Bax in MCF7 and MDA-MB-231. (Control: Cells treated with DMSO for 48; 24 h, 48 h: Cells treated with TPA for 24 and 48 h, respectively)

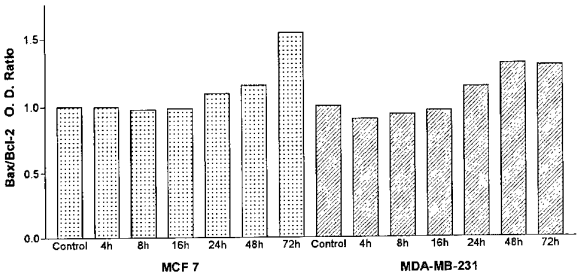

Figure 8 Alteration of ratio (in O.D. value) of Bax to Bcl-2 expression in cells treated with TPA. (Control: Cells treated with DMSO for 48; 4, 8, 16, 24, 48, 72 h: Cells treated with TPA for 4, 8, 16, 24, 48, and 72 h, respectively)

induced apoptosis (Kaufmann *et al.*, 1993; Lazebnik *et al.*, 1994; Tewari *et al.*, 1995). Additionally, flow cytometry analysis with 7AAD staining now has been conducted to detect and quantitate cells undergoing apoptosis (Philpott *et al.*, 1996; Mohammad *et al.*, 1998). Induction of apoptotic cell death by TPA in the prostate cancer cell line (LNCaP) has been shown previously. An increase in the number of apoptotic cells was first observed at 12 h, but that DNA ladder formation was initially seen at 10 h (Sundareshan *et al.*, 1997). We also found similar changes in breast cancer cells treated with TPA. DNA ladder formation and PARP cleavage were observed in breast cancer cells after 48 h of treatment with TPA, and flow cytometry revealed that the number of apoptotic cells increased with longer treatment of TPA. The decreased cell numbers in the treatment groups compared to the control group may, indeed, be due to apoptotic cell death as well as cell growth inhibition, especially in MDA-MB-231, MDA-MB-453 and SKBR3 cells. Thus, collectively, our data suggest that TPA induces apoptosis in breast cancer cells, and that the poorly differentiated cells and the cells with high metastatic ability are more sensitive to TPA treatment in the induction of apoptotic cell death.

The p53 tumor suppressor gene is known to be involved in apoptosis. Functional p53 can induce $p21^{WAF1}$ and an increased level of $p21^{WAF1}$ can decrease cyclin-dependent kinases (CDKs) activity, resulting in growth arrest (Vogelstein *et al.*, 1992; El-Deiry *et al.*, 1993; Harper *et al.*, 1993; Polyak *et al.*, 1996; Agarwal *et al.*, 1995; Xiong *et al.*, 1993). On the other hand, p53 can down-regulate Bcl-2 which allows cells to survive a variety of fatal cellular events and protects cells from apoptosis (Sano *et al.*, 1995; Hockenbery *et al.*, 1993; Kane *et al.*, 1994). This is a typical p53-dependent pathway leading to apoptosis. In our experiment, all (MDA-MB-231, MDA-MB-453, BT 20, and SKBR3) but one (MCF 7) breast cancer cell lines harbor mutant p53. The treatment of breast cancer cells with TPA did not change the level of mutant or wild-type p53 in the cells, whereas, the $p21^{WAF1}$ in all five cell lines was significantly induced by TPA. These results suggest that the induction of $p21^{WAF1}$ and apoptosis by TPA is perhaps through a p53-independent pathway. Apoptosis may be induced by either a p53-dependent or independent pathway. The up-regulation of $p21^{WAF1}$ may be one of the molecular mechanisms through which TPA induces apoptosis. An increase in $p21^{WAF1}$ results in G1 arrest in the cell cycle. Cells fail to progress to S, G2 and M phase, accumulate at the G1 checkpoint, and are destined to apoptosis.

Recent studies indicate that the proto-oncogene Bax and other related proteins (Bcl-2, Bcl-xl, etc) may play a major role in determining whether cells will undergo apoptosis under certain experimental conditions, which promote cell death. Increased expression of Bax can induce apoptosis (Sano *et al.*, 1995; Salomons *et al.*, 1997; Kitaba *et al.*, 1996; Findley *et al.*, 1997). Salomons *et al.*, (1997) found that the ratio of Bax:Bcl-2, rather than Bcl-2 alone, is important for the survival of drug-induced apoptosis in leukemic cell lines and ALL. Our data showed that there was no significant change of Bcl-2 expression and that Bax RNA and protein expressions were up-regulated in breast cancer cells treated with TPA. Thus, our results support Salomons' conclusion that the ratio of Bax and Bcl-2 protein level is important for cells undergoing apoptosis. Bax harbors a high homologic sequence of Bcl-2 and forms heterodimers with Bcl-2. Thus, Bax is a protein that antagonizes the anti-apoptotic function of Bcl-2.

Previous studies also found that TPA augmented the production of pro-matrix metalloproteinases (proMMPs) and the matrix metalloproteinases (MMPs) were upregulated by TPA (Kapila *et al.*, 1995; Cornelius *et al.*, 1995; Rawdanowicz *et al.*, 1994). MMPs are a multigene family of metal-ion-requiring proteinases, which degrade components of the extracellular matrix (ECM). Recent evidence showed that ECM suppressed apoptosis of mammary epithelial cells in tissue culture and that MMPs might be involved in inducing apoptotic death (Boudreau *et al.*, 1995; Quarrie *et al.*, 1996; Lund *et al.*, 1996), leading us to propose that induction of apoptosis by TPA might be through a MMPs-mediated pathway. Much remains to be learned about the functions of MMPs to reveal the molecular mechanisms through which TPA induces apoptosis.

In conclusion, our data shows that TPA is not only a tumor promoter but also a potent inducer of apoptosis in breast cancer cells. TPA-induced apoptosis is mediated through a p53-independent pathway.

TPA can up-regulate p21^{WAF1}, which inhibits CDKs activity and results in G1 arrest. TPA also induces Bax, which lead breast cancer cells to be destined to apoptosis.

Material and methods

Cell culture and growth inhibition

Human breast cancer cell lines (MCF7, BT20, SKBR3, MDA-MB-231 and MDA-MB-453) were obtained from ATCC and cultured in DMEM or DMEM/H12 medium (Gibco, MD, USA) supplemented with 10% FBS, 1% penicillin/streptomycin in a 5% CO2 atmosphere at 37°C. The cells were seeded at a density of 5×10^5/well in a six well culture dish. After 24 h, the cells were treated with 10 nM TPA or DMSO (vehicle control). Untreated cells and cells treated with TPA or DMSO for 24, 48, and 72 h were harvested by trypsinization, stained with 0.4% trypan blue and counted using a hemocytometer.

Protein extraction and Western blot analysis

The breast cancer cells were plated and cultured in complete medium and allowed to attach for 24 h followed by the addition of 10 nM TPA and incubation for 4, 8, 16, 24, 48 and 72 h. Control cells were incubated in the medium with DMSO for 48 h. After incubation, the cells were harvested by scraping the cells from culture dishes using a scraper and collected by centrifugation. Cells were resuspended in Tris-HCl buffer, sonicated for 2×10 s and lysed using an equal volume of 4% SDS. Protein concentration was then measured using protein assay reagents (Pierce, IL, USA). Cell extracts were boiled for 10 min and chilled on ice, subjected to 4–20% SDS–PAGE, and electrophoretically transferred to nitrocellulose membrane. Each membrane was incubated with monoclonal p21^{WAF1} (1:2000, Upstate, NY, USA), Bcl-2, p53 (1:500, Oncogene, MA, USA), Polyclonal Bax (1:500, Oncogene, MA, USA), β-actin (1:2000, Sigma, MO, USA) antibodies, washed with TTBS and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using chemiluminescent detection system (Pierce, IL, USA). Bidimentional optical densities (OD) of p21WAF1, Bcl-2, Bax, p53 and actin proteins in the films were scanned by Gel Doc 1000 (Bio-Rad, CA, USA) and quantified by Molecular Analyst software (Bio-Rad, CA, USA).

RNA extraction and Northern blot analysis

After treatment with TPA for 5, 24 and 48 h or with DMSO for 5 and 48 h, the treated and untreated cells were harvested and total RNA was extracted using TRIzol RNA extraction kit (Gibco, MD, USA). 20 μg total RNA of each sample was electrophoresed on a 1.2% agarose-formaldehyde gel. After transfer to a nylon membrane, hybridization was performed with α-^{32}P-labeled p21^{WAF1}, p53 and Bax cDNA probe. The membranes were subsequently hybridized with β-actin cDNA probe to monitor RNA loading.

DNA ladder formation

Cellular cytoplasmic DNA from cells treated with TPA for 24, 48, 72 h or with DMSO for 48 h, was extracted using 10 mM Tris (pH 8.0), 1 mM EDTA, and 0.2% Triton X-100. The lysate was centrifuged for 15 min at 13 000 g to separate the fragment DNA (soluble) from intact chromatin (nuclear pellet). The supernatant from the lysate was treated with RNase, followed by SDS-Proteinase K digestion, phenol chloroform extraction and isopropanol precipitation. DNA was separated through a 1.5% agarose gel. After electrophoresis, gels were stained with ethidium bromide and the DNA was visualized by UV light.

Analysis of cleavage of PARP

Cells treated with TPA or DMSO for 24, 48, 72 h were lysed in lysis buffer [10 mM Tris-HCl (pH 7.1), 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovanadate, 2 mM iodoacetic acid, 5 μM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100]. The lysates were kept on ice for 30 min and vigorously vortexed before centrifugation at 12 500 g for 20 min. Fifty μg of total protein were resolved on 10% SDS-PAGE and then transferred to membrane. The membrane was incubated with primary monoclonal anti-human PARP antibody (1:5000, Biomol, PA, USA), washed with TTBS and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using chemiluminescent detection system (Pierce, IL, USA).

Flow cytometry for detecting apoptosis

7-amino actinomycin D (7AAD) staining and flow cytometry was conducted to detect and quantitate apoptosis as described previously (Philpott *et al.*, 1996). Cells treated with TPA for 72 and 96 h or with DMSO for 96 h were subject to this analysis. Briefly, 7AAD (Calbiochem-Novabiochem, CA, USA) was dissolved in acetone and diluted in PBS to a concentration of 200 μg/ml. A total of 100 μl of 7AAD solution was added to 10^6 cells suspended in 1 ml PBS and mix well. Cells were stained for 20 min at 4°C while protected from light and pelleted by centrifugation. The cells were resuspended in 500 μl of 2% paraformaldehyde (PE) solution. Unstained fixed cells were used as negative control. Samples were analysed on a FACscan (Becton Dickinson, CA, USA) within 30 min of fixation. Data on 10 000 and 20 000 cells were acquired and processed using Lysys II software (Becton Dickinson, CA, USA). Scattergrams were generated by combining forward light scatter with 7AAD fluorescence, and regions were drawn around clear-cut populations having negative, dim, and bright fluorescence. The frequency of cells with low, medium, and high 7AAD fluorescence was assessed. The purity and enrichment of the sorted populations were then calculated.

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Induction of apoptosis in breast cancer cells MDA-MB-231 by genistein

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Breast cancer is the most common cancer among American women, whereas Asian women, who consume a traditional diet high in soy products, have a relatively low incidence. Genistein is a prominent isoflavonoid in soy products and has been proposed as the agent responsible for lowering the rate of breast cancer in Asian women. We investigated the effects of genistein on cell growth and apoptosis-related gene expression in breast cancer cells MDA-MB-231. We found up-regulation of Bax and p21^{WAF1} expressions and down-regulation of Bcl-2 and p53 expression in genistein-treated cells. Furthermore, DNA ladder formation, CPP32 activation, and PARP cleavage were observed after treatment with genistein, indicating apoptotic cell deaths. Flow cytometry with 7-amino actinomycin D staining showed that the number of apoptotic cells increased with longer treatment of genistein. From these results, we conclude that genistein inhibits the growth of MDA-MB-231 breast cancer cells, regulates the expression of apoptosis-related genes, and induces apoptosis through a p53-independent pathway. The up-regulation of Bax and p21^{WAF1} may be the molecular mechanisms by which genistein induces apoptosis, however, further definitive studies are needed. These results suggest that genistein may be a potentially effective chemopreventive or therapeutic agent against breast cancer.

Keywords: genistein; apoptosis; breast cancer cells

Introduction

Breast cancer is the most common cancer among American women, and remains the second leading cause of cancer related deaths in US females (Parker *et al.*, 1996). In contrast, Asian women, who consume a traditional diet high in soy products, have a relatively low incidence of breast cancer. Asians who immigrate to the United States and adopt a Western diet are at higher risk of breast cancer (Lee *et al.*, 1991; Adlercreutz *et al.*, 1991). Epidemiological studies reveal that soy-based diets containing isoflavonoid may reduce the risk of certain hormone-dependent cancers such as breast, prostate and colon cancer (Lee *et al.*, 1991; Messina *et al.*, 1991, 1994; Yuan *et al.*, 1995; Gescher *et al.*, 1998). The much lower risk of breast, colon, and prostate cancers in Asians, who consume 20–50 times more soy than Americans, has raised the question whether compounds in the soy diet act as a natural chemopreventive agent. Soy-based diets are high in isoflavonoid. Genistein is a prominent isoflavonoid in soy products and has been proposed as the agent responsible for lowering the rate of breast cancer in Asian women (Lamartiniere *et al.*, 1995; Fotsis *et al.*, 1995). The mechanism for this chemopreventive effect, however, is unknown.

Genistein has been identified as a protein tyrosine kinase (PTK) inhibitor (Akiyama *et al.*, 1987). PTKs are known to play a key role in oncogenesis, control of cell growth, and apoptosis (Hunter, 1987; Morla *et al.*, 1989; Ullrich and Schlessinger, 1990; Kyle *et al.*, 1997). It has been reported that genistein is a potent inhibitor of cell proliferation, oncogenesis and clonogenic ability in animal and human cells (Fotsis *et al.*, 1995; Barnes, 1995). Genistein also inhibits the growth of carcinogen-induced cancers in rats and human leukemia cells transplanted into mice (Lamartiniere *et al.*, 1995; Uckun *et al.*, 1995). The induction of stress proteins (e.g. glucose-related proteins and heat shock proteins) in tumor cells has been shown to protect them against apoptosis. This stress response is inhibited by genistein (Zhou and Lee, 1998). Genistein has been shown to induced apoptosis in prostate cancer and leukemia cells (Kyle *et al.*, 1997; Spinozzi *et al.*, 1994). Furthermore, studies with MCF7 breast cancer cells containing functional ER and wild type p53 also showed cell growth inhibition and apoptosis (Shao *et al.*, 1998; Wang *et al.*, 1996, 1997). The role of genistein in changing the expression of genes involved in the apoptotic pathway, however, is less well documented.

The molecular mechanism implicated in apoptosis has been partially elucidated. The induction of apoptosis is partly mediated, intracellularly by several genes, such as p53, Bcl-2, Bax, and p21^{WAF1} (Chiarugi *et al.*, 1994). The p53 tumor suppressor gene is a cell cycle regulator able to induce cell cycle arrest to allow DNA repair or apoptosis (Vogelstein and Kinzler, 1992). p21^{WAF1} is activated by the p53 protein, and an increased level of p21^{WAF1} is associated in cyclin-containing complexes with decreasing cyclin-dependent activity in damaged cells destined to apoptosis (El-Deiry *et al.*, 1993; Harper *et al.*, 1993). Although its mechanism of action is still unclear, Bcl-2 functions as a suppressor of apoptotic death triggered by a variety of signals (Sedlak *et al.*, 1995), and is negatively regulated by wild type p53. Bcl-2 overexpression has been shown to inhibit apoptosis induced by a variety of stimuli, whereas, a predominance of Bax over Bcl-2 accelerates apoptosis upon apoptotic stimuli (Williams and Smith, 1993).

The purpose of our study was to investigate the molecular mechanism by which genistein exerts its inhibitory effects on a breast cancer cell line containing mutant p53 and lacking functional ER (MDA-MB-231), and to provide the scientific rationale for using genistein as a chemopreventive or therapeutic agent against breast cancer. Our data show that genistein

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induced apoptosis with alterations of apoptosis-related gene expressions in MDA-MB-231 breast cancer cells.

Results

Effects of genistein on cell growth

The treatment of MDA-MB-231 breast cancer cells for 1–6 days with 5, 15 and 30 μM of genistein resulted in inhibition of cell proliferation. The inhibition of cell proliferation is dose-dependent. The effect of genistein on the cell growth of MDA-MB-231 is depicted in Figure 1. Inhibition of cell proliferation could be the result of the induction of apoptosis, cell cycle growth arrest and/or the inhibition of growth. We therefore, investigated whether genistein could induce apoptosis in breast cancer cells.

Induction of apoptosis

Apoptosis was observed in the cells treated with 30 μM genistein as illustrated by the DNA ladder shown in Figure 2a. The induction of apoptosis was more pronounced at 48–72 h of treatment, and is directly correlated with the inhibition of cell growth. Western blot analysis of PARP cleavage showed that the PARP protein was degraded, with the concomitant diminution of full size (116 KD) molecule and accumulation of the 85 KD, in cells after 48 h of genistein treatment (Figure 2b). Western blot analysis of CPP32 activation also showed that the CPP32 protein was cleaved to yield a 17 KD fragment after treatment of genistein (Figure 2c). These data provide strong evidence that apoptosis was induced in cells treated with genistein.

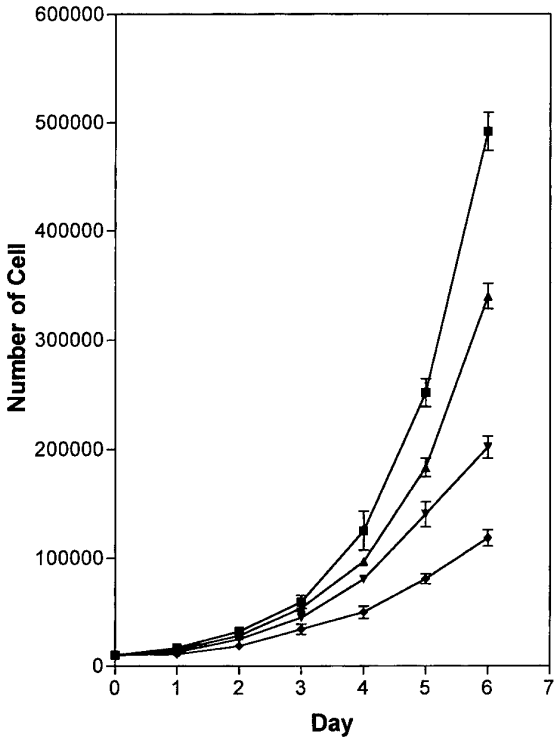

Figure 1 Cell growth inhibition by genistein

Furthermore, flow cytometry data with 7-amino actinomycin D staining showed increased apoptotic cells after genistein treatment for 24 h (13%), 48 h (29.16%) and 72 h (43.04%), compared to the control sample (7.01%) (Figure 2d). These four independent methods of measuring apoptosis provided similar results, suggesting that genistein induces apoptosis in breast cancer cells in addition to cell growth inhibition. These results, however, do not provide any evidence regarding the mechanism by which genistein induces apoptosis. In order to explore such mechanisms, we investigated the alterations in the expression of the genes involved in the apoptotic pathway.

Expression of p21^{WAF1}

The expression of p21^{WAF1} in MDA-MB-231 cells was investigated in genistein-treated and -untreated cells by Western blot analysis. The results of a typical experiment are shown in Figure 3a, which demonstrates the induction of p21^{WAF1} protein when cells were treated with 30 μM genistein for 24 h. A similar induction was also observed when cells were treated with either 5, 15 or 30 μM of genistein for 48 and 72 h. In order to obtain a quantitative value for the protein expression of p21^{WAF1}, optical density measurement was conducted as described under Materials and methods. The ratios of p21^{WAF1} to actin protein expression revealed that cells treated with genistein showed at least a 1.5-fold increase in p21^{WAF1} compared to the untreated control. The induction of p21^{WAF1} protein expression was directly correlated with the inhibition of cell growth.

Expression of Bcl-2 and Bax

The constitutive levels of Bcl-2 or Bax and the time course for the effect of genistein on Bcl-2 or Bax expression in MDA-MB-231 cells were studied by Western blot analysis. The levels of Bcl-2 expression in the MDA-MB-231 cells were slightly down-regulated with the addition of 15 or 30 μM of genistein when exposed for 24–72 h. In contrast, the expression of Bax was significantly up-regulated after 24 h of 5, 15 or 30 μM genistein treatment (Figure 3b). Optical density measurement was also conducted to obtain a quantitative value for the protein expression of Bax and Bcl-2. The ratios of Bax to Bcl-2 protein expression revealed that cells treated with 30 μM genistein showed at least an 11-fold increase in Bax compared to Bcl-2 at 24 h of treatment, ninefold at 48 h, and fourfold at 72 h (Figure 3b). These data suggest that the induction of Bax is transient and rapid, and the effect is diminished at 72 h where 43% of cells underwent apoptotic cell death.

Expression of p53

The expression of p53 in MDA-MB-231 cells with and without genistein treatment was also tested by Western blot analysis. It is important to note that MDA-MB-231 cells possess a mutation in the p53 gene (codon 280, AGA to AAA, Arg to Lys). As shown in Figure 3c, after 24 h of treatment with 30 μM genistein, the levels of mutant p53 protein in MDA-MB-231 cells decreased by about one-third. The expressions of p53 in the cells treated with 5, 15 or 30 μM genistein for 72 h were down-regulated at various degrees. This

result suggests that the down-regulation of mutant p53 may reverse the tumorigenic potential of genistein treated MDA-MB-231 cells, and further in-depth analysis is needed.

Discussion

Experimental studies have shown the inhibition of cell growth of a wide range of cultured cancer cells including leukemia, breast and prostate cancer, and lymphoma by genistein (Constantinou *et al.*, 1990; Peterson and Barnes, 1991, 1993; Buckley *et al.*, 1993). Our data also demonstrated that genistein inhibits the growth of MDA-MB-231 breast cancer cells, and that the inhibition is dose-dependent. The decreased cell numbers in the treatment groups compared to the control group may be due to apoptotic cell death as well as cell growth inhibition. Fragmentation of cellular DNA at the internucleosomal linker regions

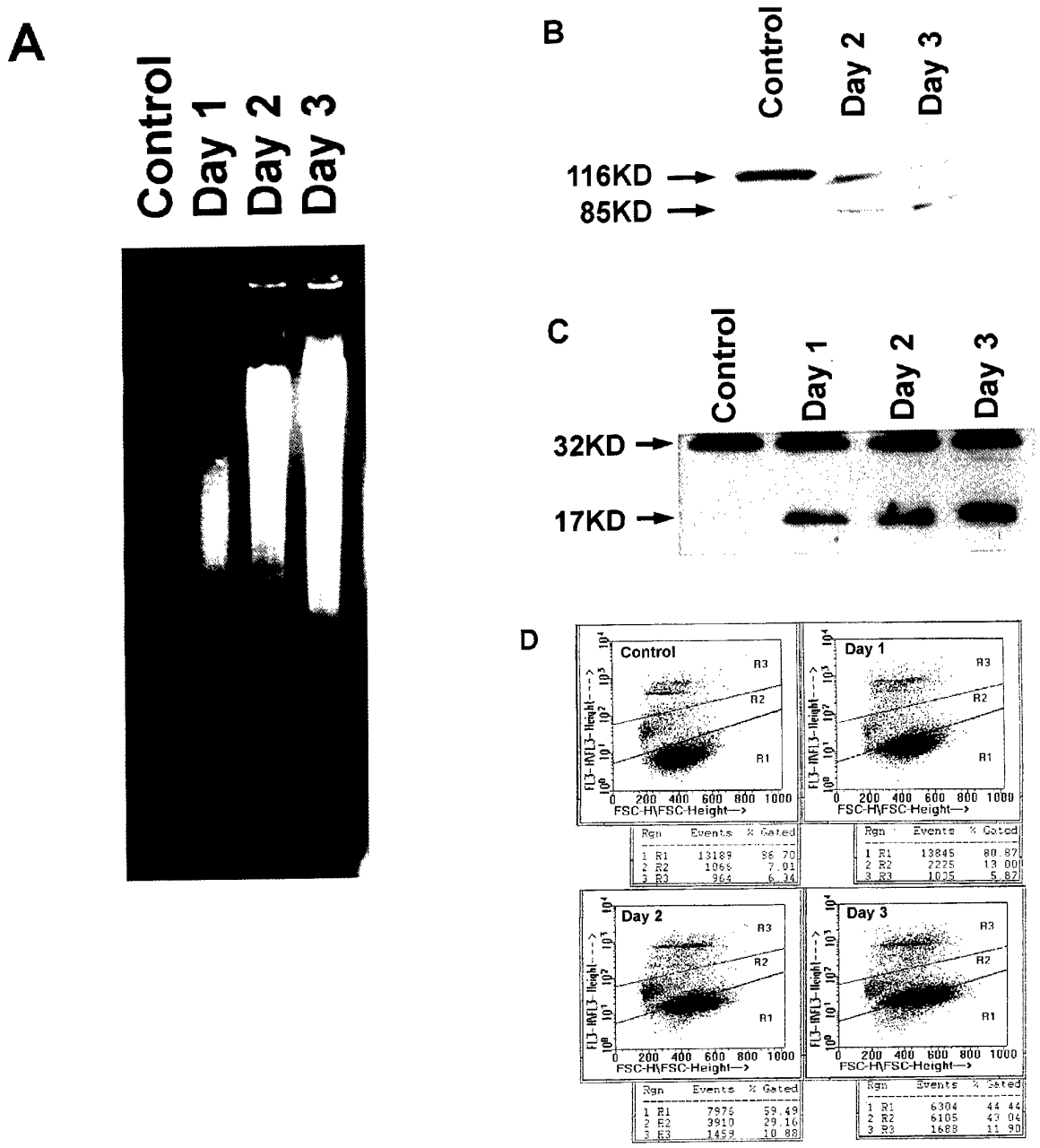

Figure 2 (**a**) DNA ladder formation in MDA-MB-231 cells treated with genistein. (Control: cells treated with Na_2CO_3 for 48 h; Day 1, Day 2, Day 3: cells treated with 30 μM genistein for 24, 48 and 72 h, respectively. (**b**) Western blot analysis of PARP cleavage. (Control: cells treated with Na_2CO_3 for 48 h; Day 2, Day 3: cells treated with 30 μM genistein for 48 and 72 h, respectively). (**c**) Western blot analysis of CPP32 activation. (Control: cells treated with Na_2CO_3 for 48 h; Day 1, Day 2, Day 3: cells treated with 30 μM genistein for 24, 48 and 72 h, respectively). (**d**) Scattergrams of 7AAD stained cells. Control: control cells treated with Na_2CO_3 for 72 h. Day 1, Day 2, Day 3: cells treated with genistein for 24, 48 or 72 h. (R1: live cells; R2: apoptotic cells; R3: dead cells)

has been observed in cells undergoing apoptosis, which was induced by a variety of agents. This cleavage produces ladders of DNA fragments that are the sizes of integer multiples of a nucleosome length (180–200 bp) (Chiarugi *et al.*, 1994; Huang *et al.*, 1997; Fisher, 1994). Because of their characteristic patterns revealed by agarose gel electrophoresis, these nucleosomal DNA ladders are widely used as biochemical markers of apoptosis. In addition, the cleavage of poly(ADP-ribose) polymerase (PARP) and the activation of Caspase-3 (CPP32) have also been used as early markers of apoptosis (Lazebnik *et al.*, 1994; Tewari *et al.*, 1995; Darmon *et al.*, 1995). Additionally, flow cytometric analysis with 7AAD staining has now been conducted to detect and quantify apoptotic cells (Philpott *et al.*, 1996). Using these techniques we, indeed, found induced apoptosis in the MDA-MB-231 breast cancer cells treated with genistein. DNA ladder formation and PARP cleavage were observed in the breast cancer cells after 48 h of treatment with genistein. CPP32 activation was also observed in the breast cancer cells after treatment with genistein. Flow cytometery revealed that the number of apoptotic cells increased from 13% (24 h of treatment) to 43% (72 h of treatment) with longer treatment of genistein). These results suggest that genistein can inhibit the growth of breast cancer cells and induce apoptosis. Our observation is consistent with studies in other cancer cell lines (Kyle *et al.*, 1997; Spinozzi *et al.*, 1994) including MCF7 breast cancer cells as discussed later.

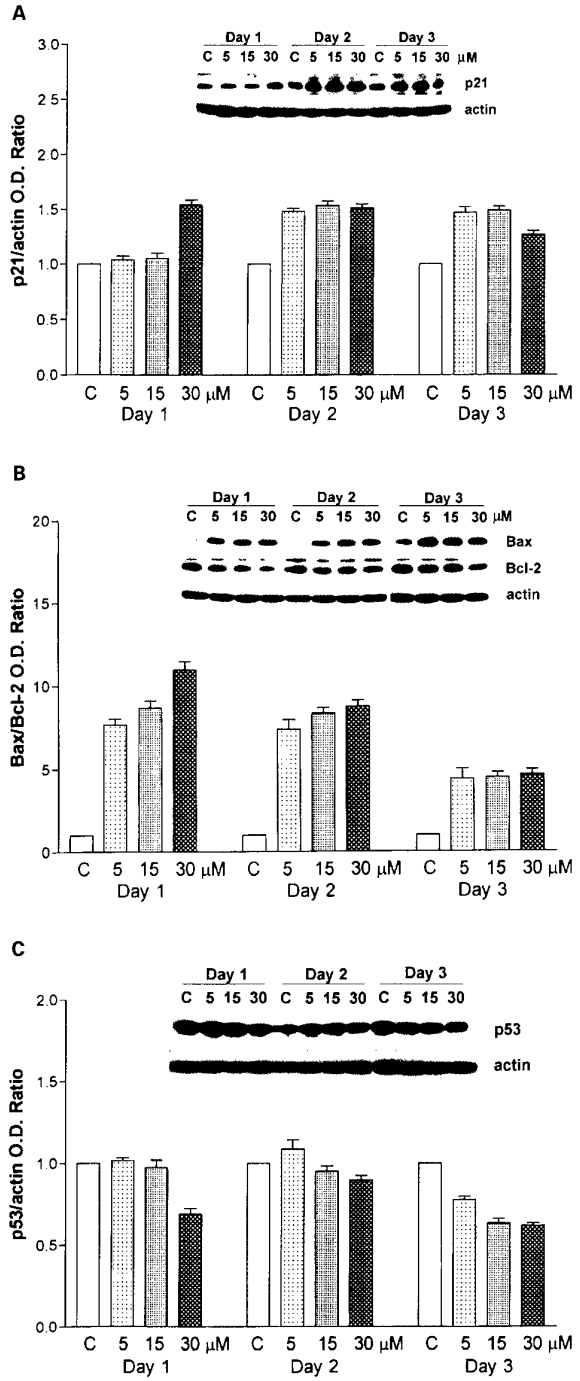

Figure 3 (**a**) Western blot and densitometric analysis of $p21^{WAF1}$ in MDA-MB-231. (Control: cells treated with Na_2CO_3; 5, 15, 30 μM: Cells treated with 5, 15, 30 μM genistein, respectively). (**b**) Wetsern blot and densitometric analysis of Bax and Bcl-2 in MDA-MB-231. (Control: cells treated with Na_2CO_3; 5, 15 and 30 μM: cells treated with 5, 15 and 30 μM genistein, respectively. (**c**) Western blot and densitometric analysis of MDA-MB-231. (Control: cells treated with Na_2CO_3; 5, 15 and 30 μM: cells treated with 5, 15 and 30 μM genistein, respectively). The bars represent the mean $\pm$ s.d. of the results from three experiments

Bax and Bcl-2 have been reported to play a major role in determining whether cells will undergo apoptosis under experimental conditions that promote cell death. Increased expression of Bax can induce apoptosis (Sano *et al.*, 1995; Salomons *et al.*, 1997; Findley *et al.*, 1997), while Bcl-2 protects cells from apoptosis (Sano *et al.*, 1995; Kane *et al.*, 1994). Salomons and associates (1997) found that the ratio of Bax : Bcl-2, rather than Bcl-2 alone, is important for the survival of drug-induced apoptosis in leukemia cell lines. Our data showed only a slight decrease of Bcl-2 expression in breast cancer cells after treatment with genistein for 48 h and no change was observed at 24 h. The expressions of Bax, however, were significantly up-regulated in breast cancer cells after treatment for 24 h. The ratio of Bax to Bcl-2 was more than 10-fold after 24 h of treatment, corresponding with a significant increase of apoptotic cells after 48 h of genistein treatment. Our results directly corroborate Salomons' conclusion that the ratio of Bax and Bcl-2 protein levels is important for cells undergoing apoptosis. Bax harbors a high homologous sequence with Bcl-2 and forms heterodimers with Bcl-2. Thus, Bax is a protein that antagonizes the anti-apoptotic function of Bcl-2. Our results suggest that up-regulation of Bax and down-regulation of Bcl-2 may be one of the molecular mechanism through which genistein induces apoptosis. However, further in-depth studies are needed in order to establish the role of Bax and Bcl-2 in genistein-induced apoptosis in this system.

The p53 and $p21^{WAF1}$ tumor suppressor genes are known to be involved in apoptosis. Functional p53 can down-regulate Bcl-2 which allows cells to survive a variety of fatal cellular events and protects cells from apoptosis (Sano *et al.*, 1995; Kane *et al.*, 1994). Functional p53 can also induce $p21^{WAF1}$, and an increased level of $p21^{WAF1}$ can decrease the activity of cyclin-dependent kinases (CDKs), resulting in growth arrest (Vogelstein *et al.*, 1992; El-Deiry *et al.*, 1993; Harper *et al.*, 1993; Agarwal *et al.*, 1995; Xiong *et al.*, 1993). Hence, functional p53 is important in p53-

dependent pathway leading to apoptosis. MDA-MB-231 breast cancer cells harbor mutant p53. The treatment of these cells with genistein down-regulated the expression of the dysfunctional p53 after treatment for 72 h, while $p21^{WAF1}$ was induced in 24 h. These results suggest that the induction of $p21^{WAF1}$ and apoptosis by genistein is through a p53-independent pathway. An increase in $p21^{WAF1}$ induced by genistein results in G2 or early M phase arrest, which was reported by our and other laboratories (Lian *et al.*, 1998; Matsukawa *et al.*, 1993). Cells fail to progress to mitosis and are destined to apoptosis. Thus, the up-regulation of $p21^{WAF1}$ may be another molecular mechanism through which genistein inhibits cancer cell growth and induces apoptosis. Some researchers have reported that genistein inhibits cell proliferation and induces apoptosis in MCF7 breast cancer cell which is ER-positive and harbors wild type p53 (Shao *et al.*, 1998; Wang *et al.*, 1996, 1997). We have similar results with MDA-MB-231 cell which is ER-negative and possesses mutant p53. Therefore, the effects of genistein on cell growth inhibition and induction of apoptosis are independent with ER and p53 status. Thus, on results, together with the results of other investigators, suggest that genistein may affect breast cancer cells irrespective of ER or p53 status.

As a potential chemopreventive or therapeutic agent against breast cancer, genistein have several advantages. It is a natural isoflavonoid derived from soy products. Other isoflavonoids in soy proteins may complement genistein in regulating gene expression and inhibiting the growth of pre-cancer and cancer cells. Genistein, even at a high concentration (200 μM), appears to have no toxic effects on mammalian tissue culture cells (Fotsis, 1995). Our results demonstrated that 5 μM genistein inhibited the growth of breast cancer cells. This concentration of genistein is nearly equivalent to the estimated *in vivo* concentration (Barnes, 1995). However, the biological role of isoflavonoids cannot be directly compared with *in vitro* experiment, particularly because human are exposed to isoflavonoids chronically, and low concentration *in vivo* may be equivalent to the higher concentration of isoflavonoids used in *in vitro* experiments, where cells are subjected to acute exposure. Thus, low concentration for a long period of time *in vivo* is equivalent to higher concentration for a short period of time *in vitro*. In conclusion, our data show that genistein inhibits the growth of breast cancer cells, regulates the expression of apoptosis-related genes, and induces apoptosis in MDA-MB-231 breast cancer cells through a p53-independent pathway. Thus, genistein may be an ideal chemopreventive or therapeutic agent for breast cancer. However, much remains to be studied about the efficacy, concentration, and mechanism of genistein as an anti-cancer agent *in vivo*.

Materials and methods

Preparation of genistein stock solution for experiments

Genistein powder (Biomol, PA, USA) was dissolved in 0.1 M Na_2CO_3 solution to make 30 mM genistein stock solution from which dilutions were made for experiments. To test any effect of Na_2CO_3 on the growth of MDA-MB-231 cells, Na_2CO_3 solution was added to the cells at the final concentration of 0.1 mM which was the highest concentration of Na_2CO_3 used in the genistein-treated cell medium. No difference of cell growth between the MDA-MB-231 cells with and without Na_2CO_3 was observed, indicating that Na_2CO_3 did not influence the experiment.

Cell culture and growth inhibition

Human breast cancer cells (MDA-MB-231) were obtained from ATCC and cultured in DMEM/F12 medium (Gibco, MD, USA) supplemented with 10% FBS, 1% penicillin/streptomycin in a 5% CO_2 atmosphere at 37°C. The cells were seeded at a density of 1×10^4/well in a six well culture dish. After 24 h, the cells were treated with 5, 15 and 30 μM of genistein or Na_2CO_3 (vehicle control). The culture medium was replaced with fresh medium and genistein or Na_2CO_3 at third day of treatment. Cells treated with genistein or Na_2CO_3 for 1 to 6 days were harvested by trypsinization, stained with 0.4% trypan blue and counted using a hemacytometer.

Protein extraction and Western blot analysis

The breast cancer cells were plated and culture in complete medium and allowed to attach for 24 h followed by the addition of 5, 15 or 30 μM genistein, and incubation for 24, 48 and 72 h. Control cells were incubated in the medium with Na_2CO_3 using same time points. After incubation, the cells were harvested by scraping the cells from culture dishes using a scraper and collected by centrifugation. Cells were resuspended in Tris-HCl buffer, sonicated for 2×10 s and lysed using an equal volume of 4% SDS. Protein concentration was then measured using protein assay reagents (Pierce, IL, USA). Cell extracts were boiled for 10 min and chilled on ice, subjected to 14 or 10% SDS–PAGE, and electrophoretically transferred to a nitrocellulose membrane. Each membrane was incubated with monoclonal $p21^{WAF1}$ (1:2000, Upstate, NY, USA), Bcl-2, p53 (1:500, Oncogene, MA, USA), Bax (1:5000, Biomol, PA, USA), and Polyclonal β-actin (1:2000, Sigma, MO, USA) antibodies, washed with TTBS and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using chemiluminescent detection system (Pierce, IL, USA).

Densitometric analysis

Autoradiograms of the Western blots were scanned with Gel Doc 1000 image scanner (Bio-Rad, CA, USA) that was linked to a Macintosh computer. The bi-dimentional optical densities (O.D.) of $p21^{WAF1}$, Bcl-2, Bax, p53 and actin proteins on the films were quantified and analyzed with Molecular Analyst software (Bio-Rad, CA, USA). The ratios of $p21^{WAF1}$/actin, Bax/Bcl-2, and p53/actin were calculated with standardizing the ratios of each control to the unit value.

DNA ladder formation

Cellular cytoplasmic DNA from cells treated with 30 μM genistein for 24, 48 and 72 h or with Na_2CO_3 for 48 h (as control), was extracted using 10 mM Tris (pH 8.0), 1 mM EDTA, and 0.2% Triton X-100. The lysate was centrifuged for 15 min at 13 000 *g* to separate the fragment DNA (soluble) from intact chromatin (nuclear pellet). The supernatant from the lysate was treated with RNase, followed by SDS-Proteinase K digestion, phenol chloroform extraction and isopropranol precipitation. DNA was separated through a 1.5% agarose gel. After electrophoresis, gels were stained with ethidium bromide and the DNA was visualized by UV light.

Analysis of cleavage of PARP

Cells treated with 30 μM genistein for 24, 48 and 74 h or with Na_2CO_3 for 48 h (as control) were lysed in lysis buffer

(10 mM Tris-HCl (pH 7.1), 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovandate, 2 mM iodoacetic acid, 5 μM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100). The lysates were kept on ice for 30 min and vigorously vortexed before centrifugation at 12 500 *g* for 20 min. Fifty μg of total protein was resolved on 10% SDS–PAGE and then transferred to nitrocellulose membrane. The membrane was incubated with primary monoclonal anti-human PARP antibody (1 : 5000, Biomol, PA, USA), washed with TTBS and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using chemiluminescence detection system (Pierce, IL, USA).

Analysis of activation of CPP32

Cells treated with 30 μM genistein for 24, 48 and 72 h or with Na_2CO_3 for 48 h (as control) were lysed in lysis buffer (10 mM Tris-HCl (pH 7.1), 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, and 1% Triton X-100). The lysates were kept on ice for 30 min and centrifuged at 12 500 *g* for 20 min. Fifty μg of total protein was resolved on 15% SDS–PAGE and then transfererd to a nitrocellulose membrane. The membrane was incubated with primary monoclonal anti-human CPP32 antibody (1 : 200, Santa Cruz, CA, USA), washed with TTBS and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using chemiluminescent detection system (Pierce, IL, USA).

Flow cytometry for detecting apoptosis

7-amino actinomycin D (7AAD) staining and flow cytometry was conducted to detect and quantify apoptosis (Philpott *et al.*, 1996). Cells treated with 30 μM genistin for 24, 48 and 72 h or with Na_2CO_3 for 72 h (as control) were subjected to this analysis. Briefly, 7AAD (Calbiochem-Novabiochem, La Jolla, CA, USA) was dissolved in acetone and diluted in PBS to a concentration of 200 μg/ml. A total of 100 μl of 7AAD solution was added to 10^6 cells suspended in 1 ml PBS and mixed well. Cells were stained for 20 min at 4°C while protected from light and pelleted by centrifugation. The cells were resuspended in 500 μl of 2% paraformaldehyde (PE) solution. Unstained fixed cells were used as negative control. Samples were analysed on a FACscan (Becton Dickinson, CA, USA) within 30 min of fixation. Data on 10 000 and 20 000 cells were acquired and processed using Lysys II software (Becton Dickinson, CA, USA). Scattergrams were generated by combining forward light scatter with 7AAD fluorescence, and regions were drawn around clear-cut populations having negative, dim, and bright fluorescence. The frequency of cells with low, medium and high 7AAD fluorescence was assessed. The purity and enrichment of the sorted populations were then calculated.

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Induction of Apoptosis and Inhibition of *c-erb*B-2 in MDA-MB-435 Cells by Genistein

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ABSTRACT

Breast cancer is the most common cancer and second leading cause of cancer related deaths in women in the United States. Genistein is a protein tyrosine kinase inhibitor and prominent isoflavonoid in soy products and has been proposed as the agent responsible for lowering the rate of breast cancer in Asian women. We have previously showed that genistein inhibits the growth of MDA-MB-231 breast cancer cells, regulates the expression of apoptosis-related genes, and induces apoptosis through a p53-independent pathway. In this study, we investigated these effects of genistein in the breast cancer cell line MDA-MB-435 and 435.eB cells that were established by transfecting *c-erb*B-2 cDNA into MDA-MB-435. We also investigated the effect of genistein on matrix metalloproteinase (MMP) secretion previously shown to be effected by *erb*B-2 transfection. Genistein was found to inhibit MDA-MB-435 and 435.eB cell growth. Induction of apoptosis was also observed in these cell lines when treated with genistein, as measured by DNA laddering, poly(ADP-ribose) polymerase (PARP) cleavage, and flow cytometric analysis. We also found an up-regulation of Bax and $p21^{WAF1}$ expression and down-regulation of Bcl-2 and *c-erb*B-2 in genistein-treated cells. Gelatin zymography showed that genistein inhibits the secretion of MMP in the breast cancer cells. From these results, we conclude that genistein inhibits the growth of MDA-MB-435 breast cancer cells, induces apoptosis, regulates the expression of genes, and may inhibit invasion and metastasis of breast cancer cells. These findings suggest that genistein may be a potentially effective chemopreventive or therapeutic agent against breast cancer.

INTRODUCTION

Breast cancer is the most common cancer in women in the United States, and remains the second leading cause of cancer related female deaths in this country [1]. In contrast, Asian women, who consume a traditional diet high in soy products, have a relatively low incidence of breast cancer [2]. Genistein is a prominent isoflavonoid in soy products and has been proposed as the agent responsible for lowering the rate of breast cancer in Asian women [3]. Genistein is a known inhibitor of protein tyrosine kinases, which play a key role in oncogenesis. Genistein has been found to inhibit cell proliferation, oncogenesis and clonogenic ability in animal and human cells [3-5]. Genistein is also known to inhibit the growth of carcinogen-induced tumors in rats, and human leukemia cells when transplanted into nude mice [6]. Genistein has recently been shown to induce apoptosis in prostate cancer, lung cancer and leukemia cells [7-9]. Furthermore, genistein has been associated with decreasing invasive and metastatic potential of cancer cells [10]. However, the molecular mechanism by which genistein exerts its multiple tumor suppressive effect has not been fully evaluated.

Apoptosis is one of the important pathways through which chemopreventive or therapeutic agents inhibit the growth of cancer cells. The induction of apoptosis is partly mediated intracellularly by several genes, such as p53, Bcl-2, Bax, and $p21^{WAF1}$ [11]. The p53 tumor suppressor gene is a cell cycle regulator able to induce cell cycle arrest to allow DNA repair or apoptosis [12]. $p21^{WAF1}$ can be activated through the p53 dependent or independent pathways, and an increased level of $p21^{WAF1}$ has been associated with a decrease in cyclin-dependent kinase activity in damaged cells destined to apoptosis [13,14]. Bcl-2 functions as a suppressor of apoptotic death triggered by a variety of

signals [15], whereas a predominance of Bax over Bcl-2 accelerates apoptosis upon apoptotic stimuli [16]. These genes have been reported to be altered by genistein in a breast cancer cell line in our recent study [17], however, their precise role requires further investigation.

Several genes have been implicated in breast cancer aggressiveness. *c-erb*B-2 is a key molecule for cancer metastasis. The *c-erb*B-2 gene encodes a glycoprotein, which is a grown factor receptor with intrinsic tyrosine kinase activity [18]. Overexpression of the *c-erb*B-2 gene has been found in approximately 20-30% of human breast cancers and the patients whose tumors overexpress *c-erb*B-2 gene have a significantly lower survival rate [19,20]. High levels of expression of *c-erb*B-2 have been positively correlated with invasion and metastasis in breast, ovarian, lung and prostate cancers [21-24]. Tan et al. found that transfection of *c-erb*B-2 into breast cancer cells increased the ability of invasion and metastasis, and that an increase of matrix metalloproteinases (MMPs) was detected in *c-erb*B-2 transfected cells [24]. MMPs have been found frequently among malignant tumor cells [25] and play an important role in tumor invasion and metastasis [26,27]. MMP-2 and MMP-9 are Type IV collagenase/gelatinase and degrade the helic domains of Type IV collagen, which is a main component of the basement membrane. These MMPs are believed to be key molecules for cancer invasion and metastasis [26-28]. Therefore, overexpression of *c-erb*B-2 is correlated with increases of MMP secretion and metastatic potential in breast cancer cells in experimental metastasis assays [24].

We have previously shown that genistein inhibits the growth of MDA-MB-231 breast cancer cells, regulates the expression of apoptosis-related genes, and induces apoptosis through a p53-independent pathway [17]. In this study, we investigated these

effects of genistein in the breast cancer cell line MDA-MB-435 and 435.eB cells that were established by transfecting *c-erb*B-2 cDNA into MDA-MB-435. We also investigated the effect of genistein on matrix metalloproteinase (MMP) secretion that was previously shown to be effected by *erb*B-2 transfection. Our data show that genistein inhibits the growth of MDA-MB-435 breast cancer cells, induces apoptosis, regulates the expression of genes, and may inhibit invasion and metastasis of breast cancer cells.

MATERIALS AND METHODS

Cell lines and culture:

Human breast cancer cell line MDA-MB-435, the 435.eB transfectant cell lines (eB1 and eB4), and the control 435.neo cell line were kindly obtained from Dr. Dihua Yu at the University of Texas M. D. Anderson Cancer Center. All the cells were cultured in DMEM/F12 medium (GIBCO, MD) supplemented with 10% FBS, 1% penicillin/streptomycin in a 5% CO_2 atmosphere at 37°C. The 435.eB transfectants were generated by transfection of the pCMV*erb*B-2 plasmid containing the 4.4 kb full-length human normal *c-erb*B-2 cDNA and the pSV2-neo plasmid carrying the neomycin-resistance selection marker gene into MDA-MB-435 cells [29]. 435.eB1 and 435.eB4 cells express 258-fold and 165-fold *c-erb*B-2 compared to parental MDA-MB-435 [29]. The control 435.neo cell line was established by transfecting the pSV2-neo plasmid alone into MDA-MB-435 cells [29].

Preparation of genistein stock solution for experiment:

Genistein powder (Biomol, PA) was dissolved in 0.1 M Na_2CO_3 solution to make 30 mM genistein stock solution from which dilutions were made for the experiments. To test any effect of Na_2CO_3 on the growth of all breast cancer cell lines, Na_2CO_3 solution was added to the cells at the final concentration of 0.1 mM which was the highest concentration of Na_2CO_3 used in the genistein-treated cell medium. No difference in cell growth between breast cancer cells with and without Na_2CO_3 was observed, indicating that Na_2CO_3 did not influence the experiment.

Cell growth inhibition:

The MDA-MB-435 cells, 435.neo cells and 435.eB cells were seeded at a density of 5×10^4/well in a six well culture dish. After 24 hours, the cells were treated with 5 μM, 15 μM, and 30 μM of genistein or Na_2CO_3 (vehicle control). Cells treated with genistein or Na_2CO_3 for one to three days were harvested by trypsinization, stained with 0.4% trypan blue and counted using a hemocytometer.

Protein extraction and Western blot analysis:

The breast cancer cells (MDA-MB-435, 435.neo, and 435.eB) were plated and cultured in complete medium and allowed to attach for 24 hours followed by the addition of 5 μM, 15 μM, or 30 μM genistein and incubation for 24, 48 and 72 hours. Control cells were incubated in the medium with Na_2CO_3 using same time points. After incubation, the cells were harvested by scraping the cells from culture dishes using a scraper and collected by centrifugation. Cells were resuspended in Tris-HCl buffer, sonicated for 2×10 sec and lysed using an equal volume of 4% SDS. Protein concentration was then measured using protein assay reagents (Pierce, IL). Cell extracts were boiled for 10 min and chilled on ice, subjected to 14 or 10 % SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane. Each membrane was incubated with monoclonal $p21^{WAF1}$ (1:2000, Upstate, NY), Bcl-2, *c-erb*B-2 (1:500, Oncogene, MA), Bax (1:5000, Biomol, PA), and Polyclonal β-actin (1:2000, Sigma, MO) antibodies, washed with TTBS and incubated with secondary antibody conjugated with

peroxidase. The signal was then detected using the chemiluminescent detection system (Pierce, IL).

RNA extraction and Northern blot analysis:

After treatment with 30 μM genistein for 4, 8, 16, 24, 48 and 72 hours, the breast cancer cells (MDA-MB-435, 435.neo, and 435.eB) were harvested and total RNA was extracted using TRIzol RNA extraction kit (Gibco, MD). 20 μg of total RNA from each sample was electrophoresed on a 1.2% agarose-formaldehyde gel. After transfer to a nylon membrane, hybridization was performed with α-^{32}P-labeled *c-erb*B-2 cDNA probe. The membranes were subsequently hybridized with β-actin cDNA probe to monitor RNA loading.

Zymography of MMP activity:

Gelatin zymography was performed according to the methods published previously [31] with a slight modification. The breast cancer cells (MDA-MB-435, 435.neo, and 435.eB) were plated and cultured in complete medium and allowed to attach overnight. The cells were washed three times with DMEM/F12 and incubated in conditioned medium for 24, 48 and 72 hours. The conditioned media were DMEM/F12 without serum and phenol red, and with 15 or 30 μM genistein or Na_2CO_3. The culture supernatants were collected and cell debris was spun off. The supernatants were concentrated using spin columns (Amicon, MA) and the protein concentration was measured using protein assay reagents (Pierce, IL). The samples with equal protein were

then subjected to 10% SDS-PAGE (containing 0.1% gelatin) without reducing agent. After electrophoresis, the gels were washed with 2.5% Triton X-100 for 30 min and with developing buffer (10 mM Tris base, 40 mM Tris HCl, 200 mM NaCl, 5mM $CaCl_2$, and 0.02% Briji 35) for one hour. After washing, the gels were incubated with fresh developing buffer overnight at 37°C. The gels were then stained with Coomassie solution (0.5% Coomassie blue in 10% methanol, 5% acetic acid) for one hour and destained with same solution without Coomassie blue. The gels were photographed and scanned for densitometric analysis.

Densitometric analysis:

Autoradiograms of the Western blots and zymograph gels were scanned with Gel Doc 1000 image scanner (Bio-Rad, CA) linked to a Macintosh computer. The bi-dimentional optical densities (O. D.) of $p21^{WAF1}$, Bcl-2, Bax, *c-erb*B-2, and actin proteins on the films and MMP on gels were quantified and analyzed with Molecular Analyst software (Bio-Rad, CA). The Ratios of $p21^{WAF1}$/actin, Bax/Bcl-2, *c-erb*B-2/actin, and MMP were calculated with standardizing the ratios of each control to the unit value.

DNA ladder formation:

Cellular cytoplasmic DNA from cells treated with 30 μM genistein for 24, 48, 72 h or with Na_2CO_3 for 48 h (as control), was extracted using 10 mM Tris (pH 8.0), 1mM EDTA, and 0.2% Triton X-100. The lysate was centrifuged for 15 min at 13,000×g to separate the fragment DNA (soluble) from intact chromatin (nuclear pellet). The supernatant from the lysate was treated with RNase, followed by SDS-Proteinase K

digestion, phenol chloroform extraction and isopropanol precipitation. DNA was separated through a 1.5% agarose gel. After electrophoresis, gels were stained with ethidium bromide and the DNA was visualized by UV light.

Analysis of cleavage of PARP:

Cells treated with 30 μM genistein for 24, 48, 72 hours or with Na_2CO_3 for 48 hours (as control) were lysed in lysis buffer [10 mM Tris-HCl (pH 7.1), 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovandate, 2 mM iodoacetic acid, 5 μM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X100]. The lysates were kept on ice for 30 min and vigorously vortexed before centrifugation at 12,500 X g for 20 min. Fifty μg of total protein was resolved on 10% SDS-PAGE and then transferred to nitrocellulose membrane. The membrane was incubated with primary monoclonal anti-human PARP antibody (1:5000, Biomol, PA), washed with TTBS and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (Pierce, IL).

Flow cytometry for detecting apoptosis:

7-amino actinomycin D (7AAD) staining and flow cytometry were conducted to detect and quantify apoptosis [30]. Cells treated with 30 μM genistein for 24, 48, 72 h or with Na_2CO_3 for 72h (as control) were subjected to this analysis. Briefly, 7AAD (Calbiochem-Novabiochem, La Jolla, CA) was dissolved in acetone and diluted in PBS to a concentration of 200 μg/ml. A total of 100 μl of 7AAD solution was added to 10^6 cells

suspended in 1 ml PBS and mixed well. Cells were stained for 20 minutes at 4°C while protected from light and pelleted by centrifugation. The cells were resuspended in 500 μl of 2% paraformaldehyde (PE) solution. Unstained fixed cells were used as negative control. Samples were analyzed on a FACscan (Becton Dickinson, CA) within 30 minutes of fixation. Data on 10,000 and 20,000 cells were acquired and processed using Lysys II software (Becton Dickinson, CA). Scattergrams were generated by combining forward light scatter with 7AAD fluorescence, and regions were drawn around clear-cut populations having negative, dim, and bright fluorescence. The frequency of cells with low, medium, and high 7AAD fluorescence was assessed. The purity and enrichment of the sorted populations were then calculated.

RESULTS

Effects of genistein on cell growth:

The treatment of MDA-MB-435, 435.neo, 435.eB1 and 435.eB4 breast cancer cells for 1-3 days with 5 μM, 15 μM, and 30 μM of genistein resulted in inhibition of cell proliferation, which was dose-dependent. The effect of genistein on the cell growth of MDA-MB-435 and 435 transfectants is depicted in Fig. 1. There appeared to be more growth inhibition in 435.eB1 cells, which express more *c-erb*B-2, than in other cell lines expressing less *c-erb*B-2. While the difference was not statistically significant, slightly more inhibition was correlated with *erb*B-2 expression. The inhibition of cell proliferation, however, could be due to the induction of apoptosis elicited by genistein. We, therefore, investigated whether genistein could induce apoptosis in these breast cancer cells.

Induction of Apoptosis:

Apoptosis was tested in all the cell lines treated with 30 μM genistein as demonstrated by the DNA ladder shown in Fig. 2A. The induction of apoptosis was more pronounced at 48-72 hours of treatment, and is directly correlated with the inhibition of cell growth. PARP cleavage analysis showed that the full size PARP (116KD) protein was cleaved to yield an 85 KD fragment after treatment with genistein (Fig. 2B) in all cell lines tested. Furthermore, flow cytometric analysis with 7-amino actinomycin D staining showed increased apoptotic cells after genistein treatment for 24 hours (9.77%), 48 hours (24.22%) and 72 hours (46.35%) in 435.eB1 cells, compared to the control cells (1.67%) [Fig. 2C]. There were similar results in other cell lines tested. 435.eB1 cells

showed more apoptotic cells after genistein treatment for 3 days compared to other cell lines expressing less *c-erb*B-2. These three independent methods of measuring apoptosis provided strong evidence that apoptosis was induced in all cell lines treated with genistein. In order to explore the mechanisms by which genistein induces apoptosis, we investigated the alterations in the expression of the genes involved in the apoptotic pathway.

Expression of $p21^{WAF1}$:

The expression of $p21^{WAF1}$ in MDA-MB-435, 435.neo, and 435.eB cells was investigated in genistein-treated and -untreated cells by Western blot analysis. The results of typical experiments are shown in Fig. 3A, which demonstrates the induction of the $p21^{WAF1}$ protein when the cells were treated with genistein for 24 hours. In order to obtain a quantitative value for the protein expression of $p21^{WAF1}$, optical density measurement was conducted as described under materials and methods. The ratios of $p21^{WAF1}$ to actin protein expression revealed that cells treated with genistein showed at least a sevenfold increase in $p21^{WAF1}$ compared to the untreated control. The induction of $p21^{WAF1}$ protein expression was directly correlated with the inhibition of cell growth. No difference of $p21^{WAF1}$ expression among these 435 cell lines was observed, suggesting that *c-erb*B-2 expression level may not contribute to the induction of $p21^{WAF1}$.

Expression of Bcl-2 and Bax:

The constitutive levels of Bcl-2 or Bax and the time course for the effect of genistein on Bcl-2 or Bax expression in MDA-MB-435, 435.neo, and 435.eB cells were

studied by Western blot analysis. The levels of Bcl-2 expression in all 435 cell lines were slightly down-regulated with the addition of genistein when exposed for 24 to 72 hours. In contrast, the expression of Bax was significantly up-regulated after 24 hours of 15 μM or 30 μM genistein treatment (Fig. 3B). Optical density measurement was also conducted to obtain a quantitative value for the protein expression of Bax and Bcl-2. The ratios of Bax to Bcl-2 protein expression revealed that cells treated with 30 μM genistein showed at least a two-fold increase in Bax compared to Bcl-2 at 24 hours of treatment, and three-fold at 48 and 72 hours (Fig. 3B). No difference of Bcl-2 and Bax expression among these 435 cell lines was observed, suggesting that the modulation in Bax and Bcl-2 by genistein is not dependent on *c-erb*B-2 expression.

Expression of *c-erb*B-2:

In order to explore the effect of genistein on *c-erb*B-2 that has been related with metastatic potential, the expression of *c-erb*B-2 in MDA-MB-435 and 435 transfectant cells with and without genistein treatment was tested by Northern and Western blot analysis. As Tan et al. reported [24], there were higher expressions of *c-erb*B-2 in 435.eB1 cells and 435.eB4 cells than in parental MDA-MB-435 and 435.neo cells. As shown in Fig. 4A and 4B, the levels of *c-erb*B-2 mRNA in 435.eB1 cells decreased after 16 hours of treatment with 30 μM genistein, and *c-erb*B-2 protein significantly decreased by about 50% after treatment with genistein for 24 hours. The expression of *c-erb*B-2 in other cell lines treated with 5 μM, 15 μM, or 30 μM genistein was also down-regulated at various degrees (Fig. 4B).

Enzyme activities of MMPs:

The activities of the basement membrane-degrading MMPs in MDA-MB-435 and 435 transfectants with or without genistein treatment were measured by zymographic analysis. Significantly higher levels of MMP-2 (72KD) and MMP-9 (92KD) were detected in the conditioned medium of the 435.eB1 and 435.eB4 transfectants than in parental MDA-MB-435 and 435.neo, while decreasing levels of MMPs were observed in the conditioned medium of all 435 cells treated with genistein (Fig. 4C). These data suggested that increased *c-erb*B-2 expression in 435.eB cells can lead to increased secretion of MMPs as reported earlier [24], and that genistein can inhibit the expression of *c-erb*B-2 and, therefore, decrease the secretion of MMPs.

DISCUSSION

It has been reported that genistein inhibits cell growth of a wide range of cultured cancer cells including breast, lung and prostate cancers, leukemia, and lymphoma [31-33]. Our previous experiment also demonstrated the growth inhibition of MDA-MB-231 breast cancer cells by genistein [17]. In the current study, we also found that genistein can inhibit the growth of MDA-MB-435, 435.neo and 435.eB transfectants. The inhibition of cell growth observed in genistein-treated cells may be partly due to apoptotic cell death apart from cell cycle arrest, previously showed by many investigators [10,34]. Thus, we employed several methods to detect apoptosis in our system. The nucleosomal DNA ladder has been widely used as biochemical markers of apoptosis for several years [35]. In addition, the cleavage of poly(ADP-ribose) polymerase (PARP) has also been used as early markers of apoptosis [36]. Additionally, flow cytometric analysis with 7AAD staining has been conducted to detect and quantify apoptotic cells [8,30,37]. Using these various techniques we found induced apoptosis in the MDA-MB-435 and 435.eB transfectants treated with genistein. DNA ladder formation and PARP cleavage were observed in the breast cancer cells after treatment with genistein. Flow cytometry revealed that the number of apoptotic cells increased from 9.77 (24 hours of treatment) to 46.36% (72 hours of treatment) with longer treatment of genistein. These results are consistent with our previous study in MDA-MB-231 cells [17] and are consistent with other studies reported in the literature [7,9]. Thus, our results clearly suggest that genistein can inhibit the growth of breast cancer cells and induce apoptosis.

It has been demonstrated that Bcl-2 and Bax play a major role in determining whether cells will undergo apoptosis under experimental conditions that promote cell

death. Bcl-2 protects cells from apoptosis [38], while increased expression of Bax can induce apoptosis [16,38]. It has also been found that the ratio of Bax:Bcl-2, rather than Bcl-2 alone, is important for the survival of drug-induced apoptosis in leukemia cell lines [16]. In our study, only a slight decrease in Bcl-2 expression was observed in breast cancer cells after treatment with genistein. The expression of Bax, however, was significantly up-regulated in breast cancer cells after treatment for 24 hours. The ratio of Bax to Bcl-2 was more than two-fold after 24 hours of treatment, corresponding with a significant increase of apoptotic cells after 48 hours of genistein and indicating that the ratio of Bax and Bcl-2 protein levels is important for cells undergoing apoptosis. These changes, however, were not related with the status of *c-erb*B-2 expression. Our results suggest that up-regulation of Bax and down-regulation of Bcl-2 may be one of the molecular mechanisms through which genistein induces apoptosis. The $p21^{WAF1}$ gene has been identified to be involved in apoptosis as well. $p21^{WAF1}$can be activated and induced through p53 dependent or independent pathways, and an increased level of $p21^{WAF1}$ was found to decrease the activity of cyclin-dependent kinases (CDKs), resulting in growth arrest and apoptosis [13,14]. In this study, $p21^{WAF1}$ was significantly induced after genistein treatment for 24 hours in all 435 cells regardless of the status of *c-erb*B-2 expression. Our previous studies, along with studies reported by other investigators, showed that the induction of $p21^{WAF1}$ and apoptosis by genistein was thought to be through a p53-independent pathway [8,10,17]. An increase in $p21^{WAF1}$ induced by genistein, results in G2 or early M phase arrest reported by us and others [8,34]. Cells failing to progress to mitosis are, thus, destined to apoptosis by genistein treatment. Therefore, the up-regulation of $p21^{WAF1}$ may be another molecular mechanism through

which genistein inhibits cancer cell growth and induces apoptosis. However, further in-depth studies are needed to establish the role of Bax, Bcl-2 and $p21^{WAF1}$ in genistein induced apoptosis.

*c-erb*B-2 has been found to be overexpressed in many cancers, especially in breast cancer [18-20]. Overexpression of *c-erb*B-2 has been correlated with lymph node metastasis in breast cancer patients [21]. In earlier studies, Yu et al [24,29] introduced the human *c-erb*B-2 gene into the very low *c-erb*B-2-expressing MDA-MB-435 human breast cancer cells and established 435.eB transfectants that express high levels of *c-erb*B-2. They compared the metastatic phenotypes of parental MDA-MB-435 cells and the 435.eB transfectants, and found that overexpression of *c-erb*B-2 can enhance the MMPs secretion and metastatic potential of MDA-MB-435 human breast cancer cells. In our study, the 435.eB transfectants and parental MDA-MB-435 cells were treated with genistein. We found that genistein inhibited *c-erb*B-2 expression in all of the cell lines tested. The *c-erb*B-2 protein is a growth factor receptor with tyrosine kinase activity. Genistein has been identified as an inhibitor of protein tyrosine kinase. Our results suggest that genistein may inhibit growth of breast cancer cells through inhibition of *c-erb*B-2 expression and its tyrosine kinase activity. Since MMPs were previously found to be upregulated by *c-erb*B-2 transfection and overexpression, we compared the secretion of MMPs in 435.eB transfectants and MDA-MB-435 with and without genistein treatment. Higher levels of MMPs in 435.eB transfectants than in parental MDA-MB-435 were observed, corresponding with a higher metastatic potential of 435.eB transfectants as previously reported [24]. After treatment with genistein, the levels of MMPs decreased in 435.eB transfectants and MDA-MB-435 parental cells. Shao et al. recently reported

similar results showing that genistein inhibited MMPs secretion in MCF7 and MDA-MB-231 breast cancer cells [10]. Our results suggest that genistein can inhibit the expression of *c-erb*B-2 and, in turn, decrease the secretion of MMPs in breast cancer cells, and further suggest that genistein may inhibit *c-erb*B-2 induced invasive and metastatic properties of breast cancer cells.

In conclusion, our data show that genistein inhibits the growth of breast cancer cells, regulates the expression of apoptosis-related genes, and induces apoptosis in MDA-MB-435 breast cancer cells. Furthermore, genistein inhibits the expression of *c-erb*B-2 and the secretion of MMPs, and may inhibit invasion and metastasis of breast cancers. Thus, genistein may be an effective chemopreventive or therapeutic agent for breast cancer. However, much remains to be studied about the molecular mechanisms of genistein as an anti-cancer agent *in vivo*.

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Figure Legends:

Figure 1. Cell growth inhibition by genistein.

Figure 2.

A. DNA ladder formation in MDA-MB-435 cells and 435.eB transfectants treated with genistein. (Control: cells treated with Na_2CO_3 for 48h; Day 1, Day 2, Day 3: cells treated with 30 μM genistein for 24, 48, 72 hours, respectively)

B. Western blot analysis of PARP cleavage in MDA-MB-435 cells and 435.eB transfectants treated with genistein. (Control: cells treated with Na_2CO_3; Day 1, Day 2, Day 3: cells treated with 30 μM genistein for 24, 48, 72 hours, respectively)

C. Scattergrams of 7AAD stained cells. Control: Control cells treated with Na_2CO_3 for 72 hours. Day 1, Day 2, Day 3: Cells treated with genistein for 24, 48, or 72 hours. (R1: dead cells; R2: apoptotic cells; R3: live cells)

Figure 3.

A. Western blot and densitometric analysis of $p21^{WAF1}$ in MDA-MB-435 cells and 435.eB transfectants treated with genistein. (C: Cells treated with Na_2CO_3; 5, 15, 30 μM: Cells treated with 5, 15, 30 μM genistein, respectively)

B. Western blot and densitometric analysis of Bax and Bcl-2 in MDA-MB-435 cells and 435.eB transfectants treated with genistein. (C: Cells treated with Na_2CO_3; 5, 15, 30 μM: Cells treated with 5, 15, 30 μM genistein, respectively)

Figure 4.

A. Northern blot and densitometric analysis of *c-erb*B-2 in 435.eB1 and MDA-MB-435 cells treated with genistein. (C: Cells treated with Na_2CO_3 for 48 hours; 4, 8, 16, 24, 48, 72h: Cells treated with 30 μM genistein for 4, 8, 16, 24, 48, 72 hours, respectively)

B. Western blot and densitometric analysis of *c-erb*B-2 in MDA-MB-435 cells and 435.eB transfectants treated with genistein. (C: Cells treated with Na_2CO_3; 5, 15, 30 μM: Cells treated with 5, 15, 30 μM genistein, respectively)

C. Zymographic and densitometric analysis of MMPs in MDA-MB-435 cells and 435.eB transfectants treated with genistein. (C: Cells treated with Na_2CO_3; 15, 30 μM: Cells treated with 15, 30 μM genistein, respectively)

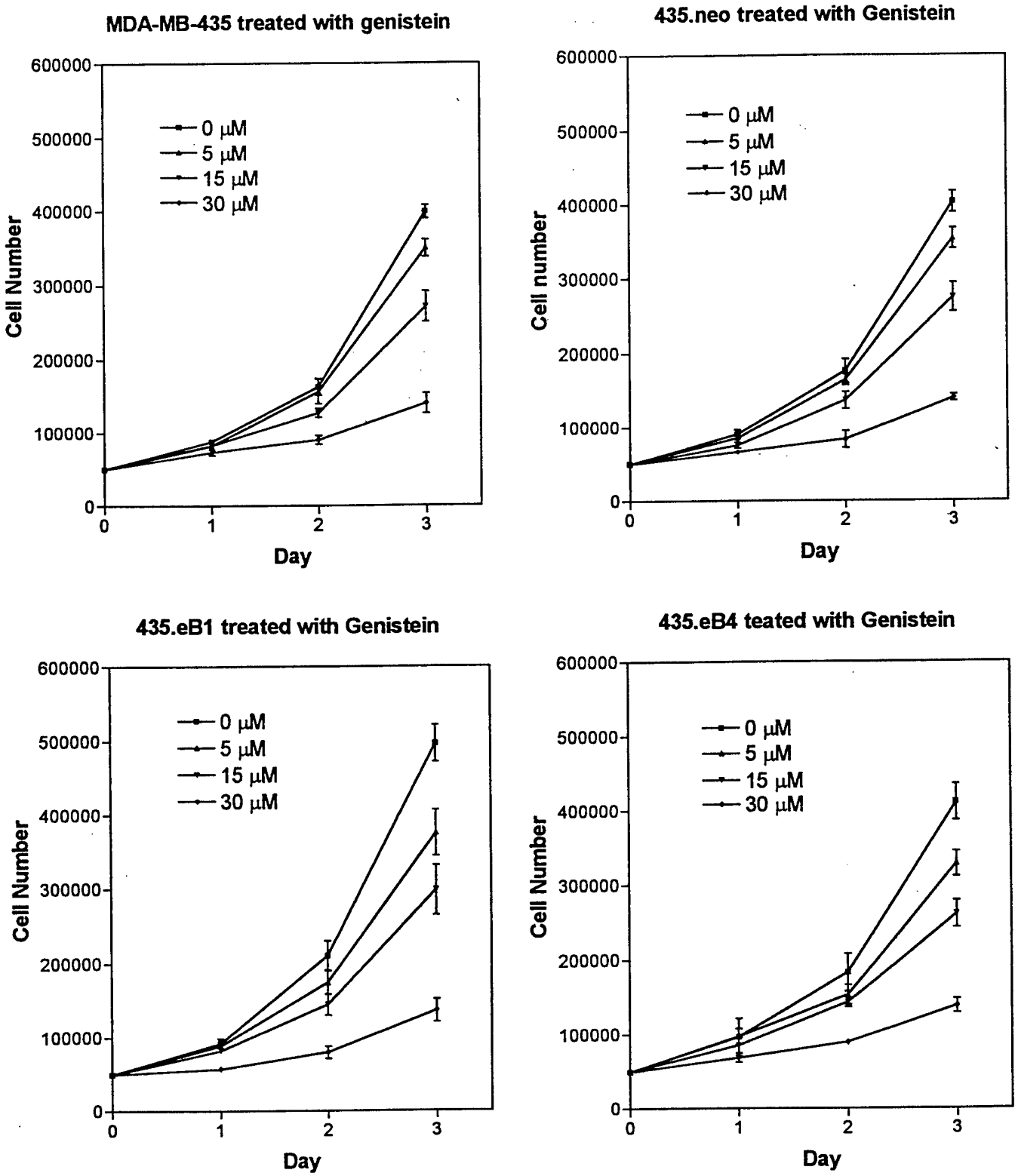

MDA-MB-435 treated with genistein
Cell Number
Day
0 μM
5 μM
15 μM
30 μM
435.neo treated with Genistein
Cell number
Day
0 μM
5 μM
15 μM
30 μM
435.eB1 treated with Genistein
Cell Number
Day
0 μM
5 μM
15 μM
30 μM
435.eB4 teated with Genistein
Cell Number
Day
0 μM
5 μM
15 μM
30 μM

Figure 1

Control
Day 1
Day 2
Day 3
Control
Day 1
Day 2
Day 3
Control
Day 1
Day 2
Day 3
435.eB1
435.eB4
MDA-MB-435

Figure 2A

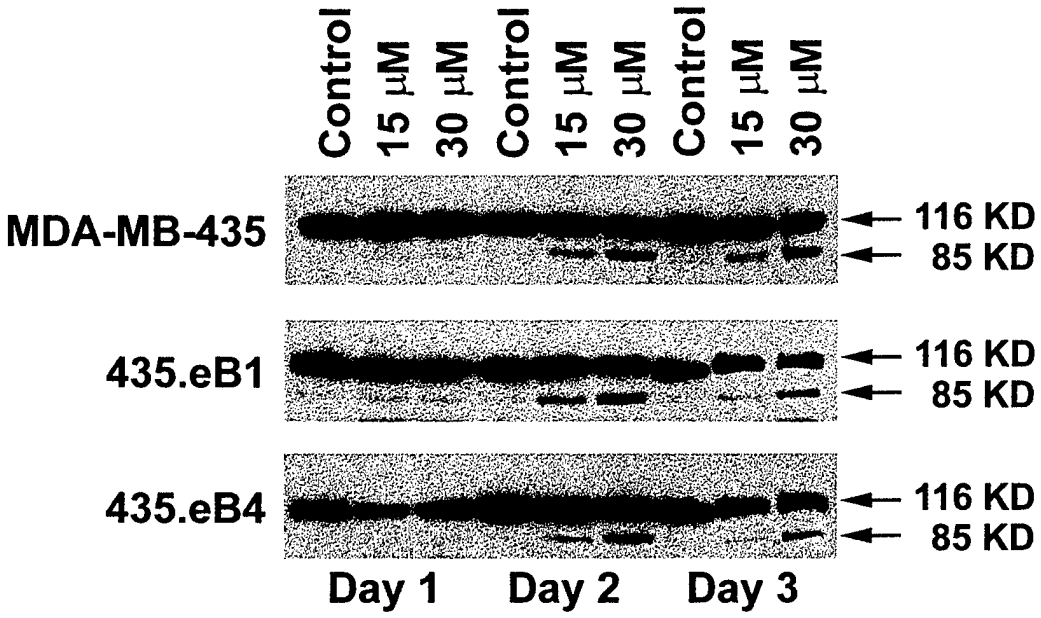
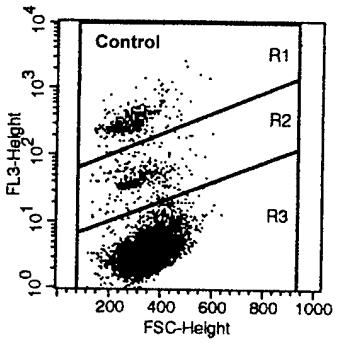
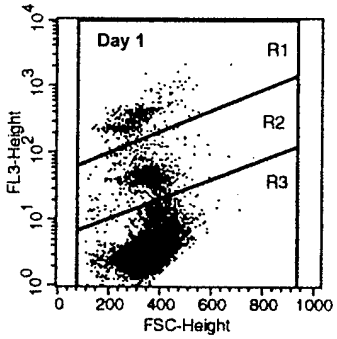

Control
15 μM
30 μM
Control
15 μM
30 μM
Control
15 μM
30 μM
MDA-MB-435
435.eB1
435.eB4
116 KD
85 KD
116 KD
85 KD
116 KD
85 KD
Day 1
Day 2
Day 3

Figure 2B

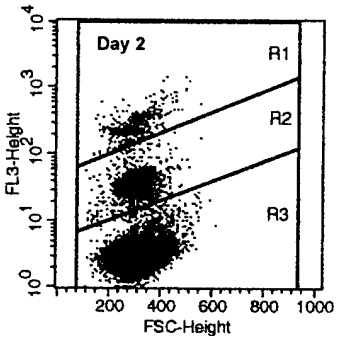
MDA-MB-435



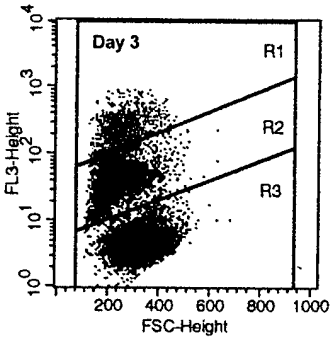
R1 3.06% R2 1.52% R3 95.42%



R1 2.99% R2 8.17% R3 88.84%

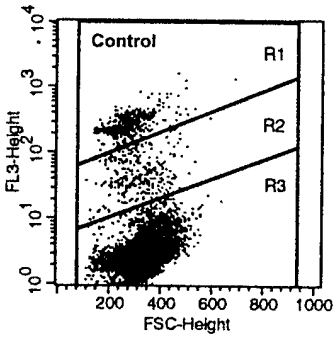


R1 5.67% R2 20.44% R3 67.89%

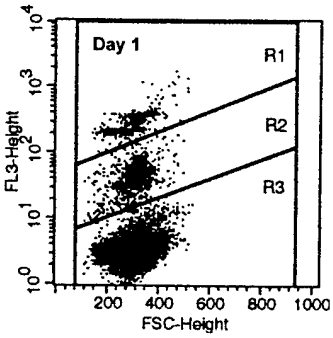


R1 8.62% R2 41.91% R3 49.47%

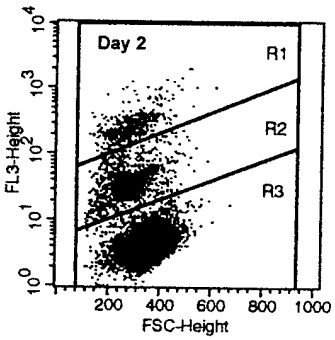
435.eB1



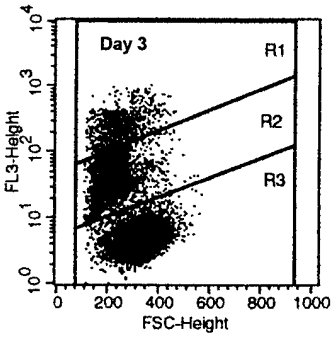
R1 2.18% R2 1.67% R3 96.15%



R1 3.52% R2 9.77% R3 86.71%

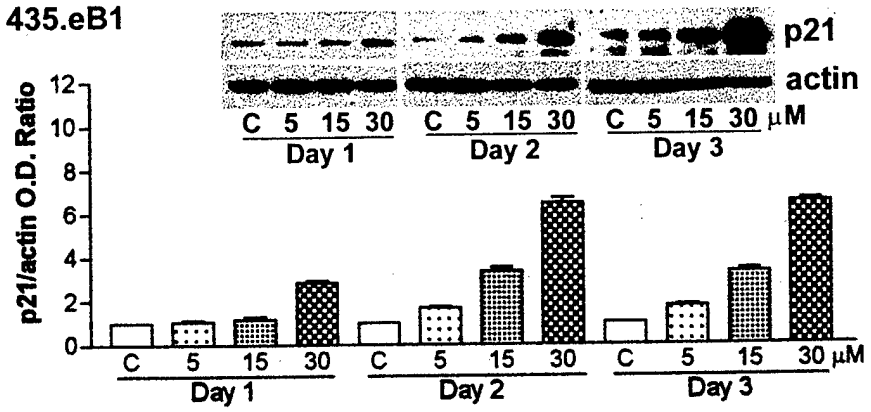


R1 5.11% R2 24.22% R3 69.67%



R1 8.01% R2 46.35% R3 45.64%

Figure 2C


435.eB1
p21
actin
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1
Day 2
Day 3
p21/actin O.D. Ratio
12
10
8
6
4
2
0
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1
Day 2
Day 3

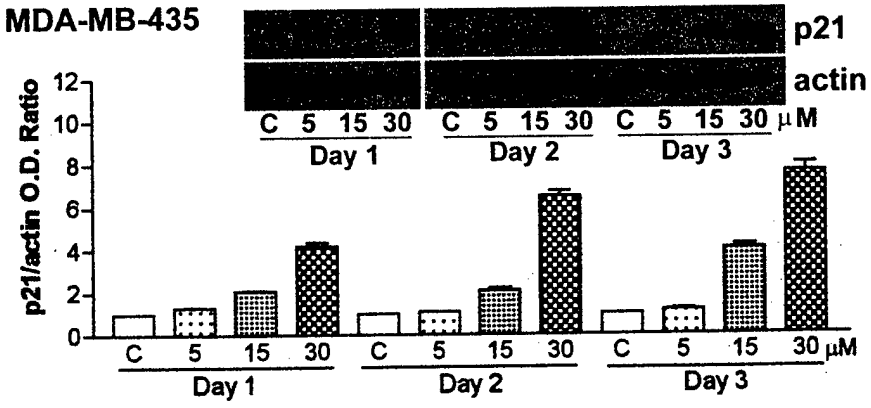

MDA-MB-435
p21
actin
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1
Day 2
Day 3
p21/actin O.D. Ratio
12
10
8
6
4
2
0
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1
Day 2
Day 3

Figure 3A

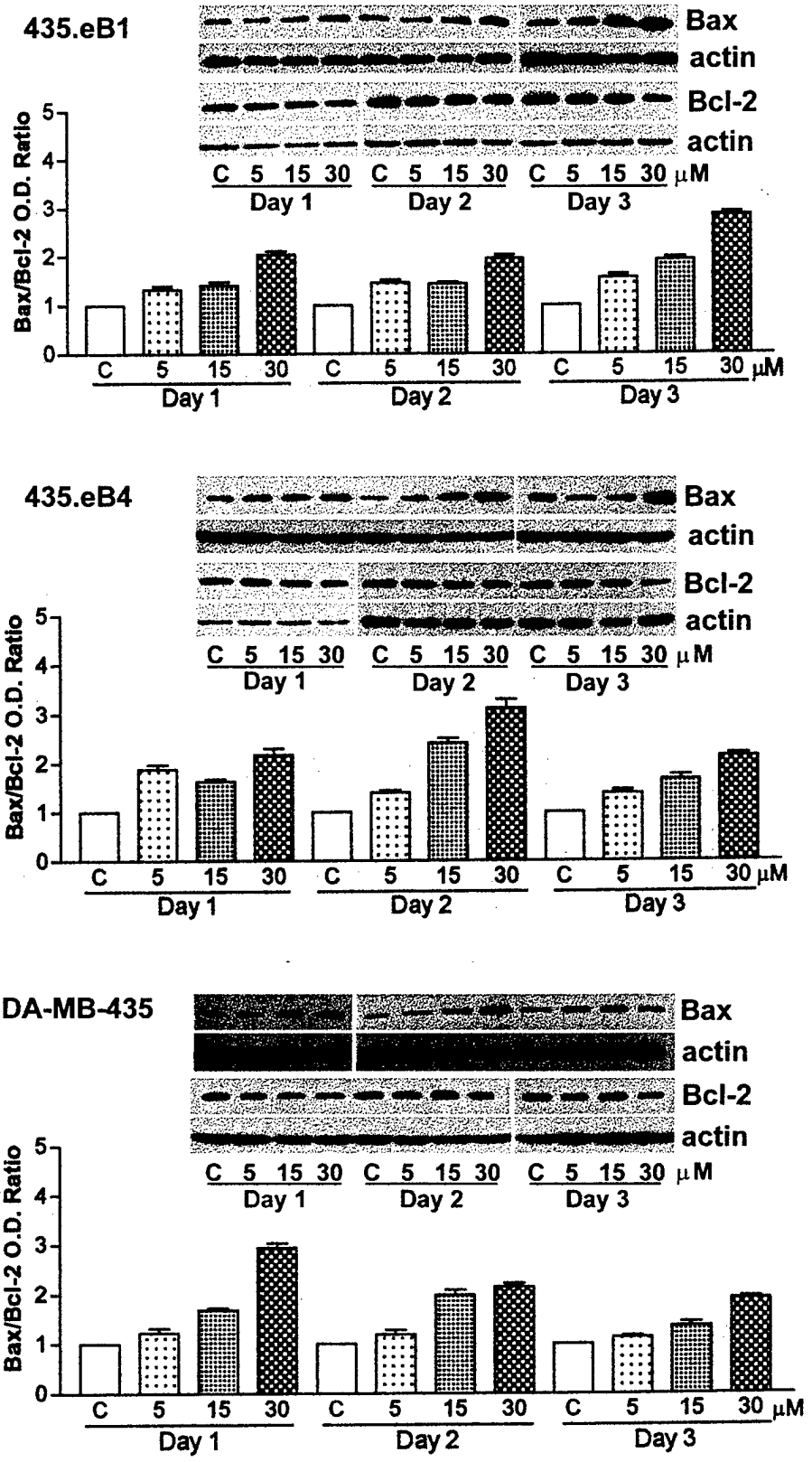

435.eB1
Bax
actin
Bcl-2
actin
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1 Day 2 Day 3
Bax/Bcl-2 O.D. Ratio
0 1 2 3 4 5
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1 Day 2 Day 3
435.eB4
Bax
actin
Bcl-2
actin
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1 Day 2 Day 3
Bax/Bcl-2 O.D. Ratio
0 1 2 3 4 5
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1 Day 2 Day 3
MDA-MB-435
Bax
actin
Bcl-2
actin
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1 Day 2 Day 3
Bax/Bcl-2 O.D. Ratio
0 1 2 3 4 5
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1 Day 2 Day 3

Figure 3B

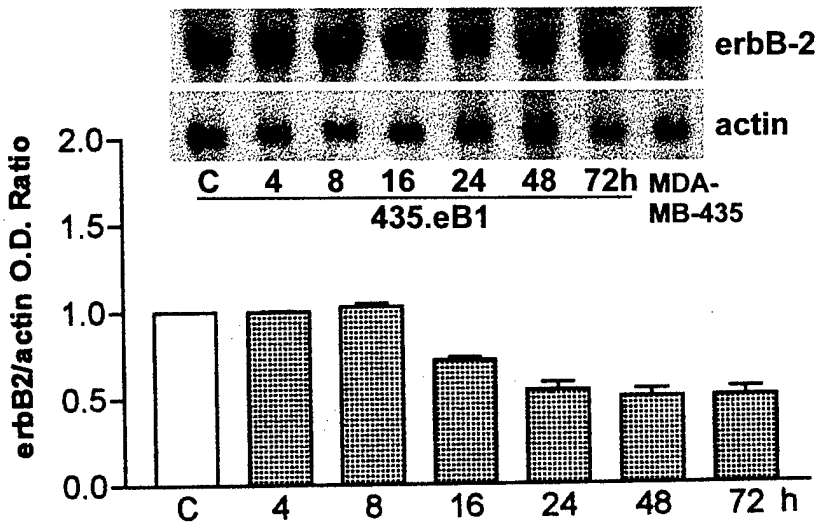

erbB-2
actin
C 4 8 16 24 48 72h
435.eB1
MDA-MB-435
erbB2/actin O.D. Ratio
2.0
1.5
1.0
0.5
0.0
C 4 8 16 24 48 72 h

Figure 4A

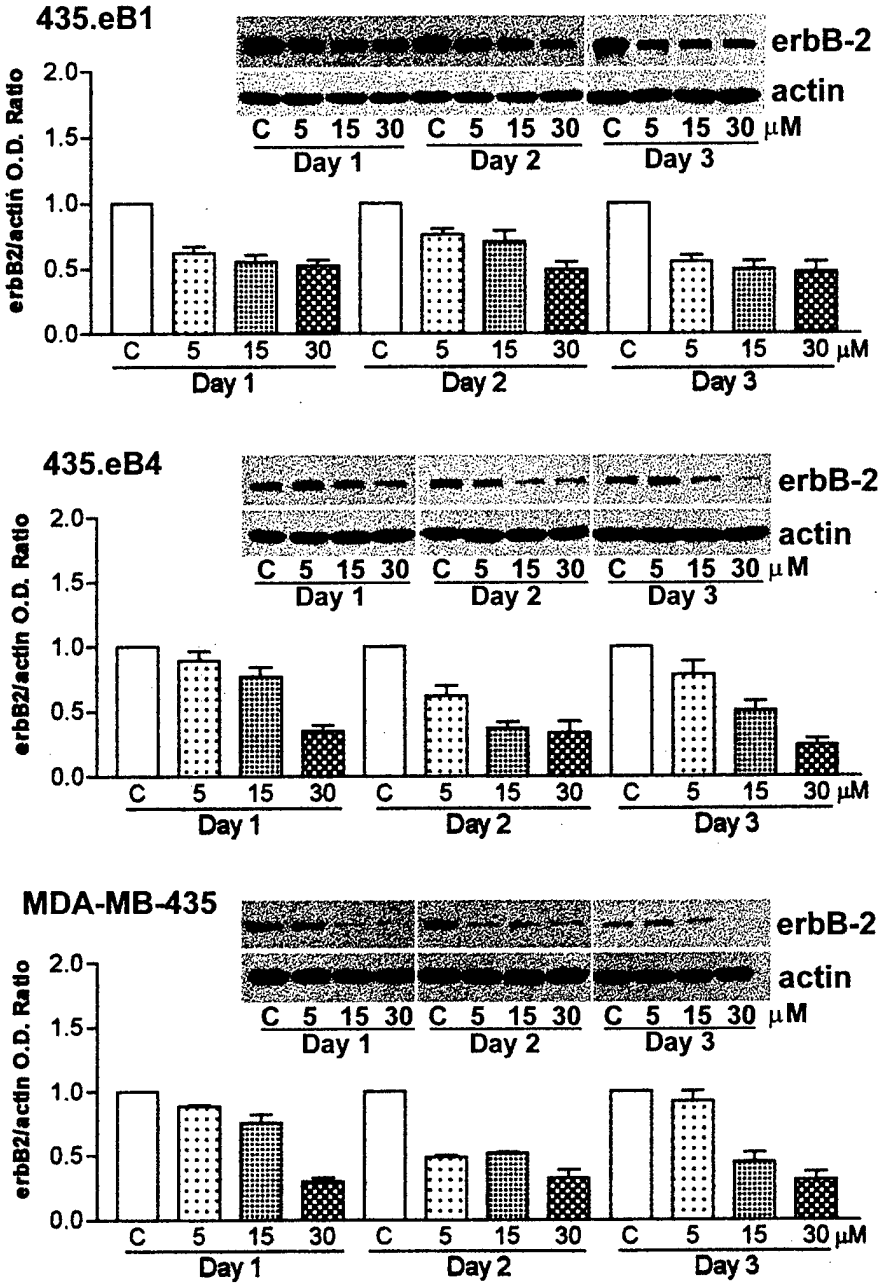

435.eB1
erbB-2
actin
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1
Day 2
Day 3
erbB2/actin O.D. Ratio
2.0
1.5
1.0
0.5
0.0
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1
Day 2
Day 3
435.eB4
erbB-2
actin
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1
Day 2
Day 3
erbB2/actin O.D. Ratio
2.0
1.5
1.0
0.5
0.0
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1
Day 2
Day 3
MDA-MB-435
erbB-2
actin
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1
Day 2
Day 3
erbB2/actin O.D. Ratio
2.0
1.5
1.0
0.5
0.0
C 5 15 30 C 5 15 30 C 5 15 30 μM
Day 1
Day 2
Day 3

Figure 4B

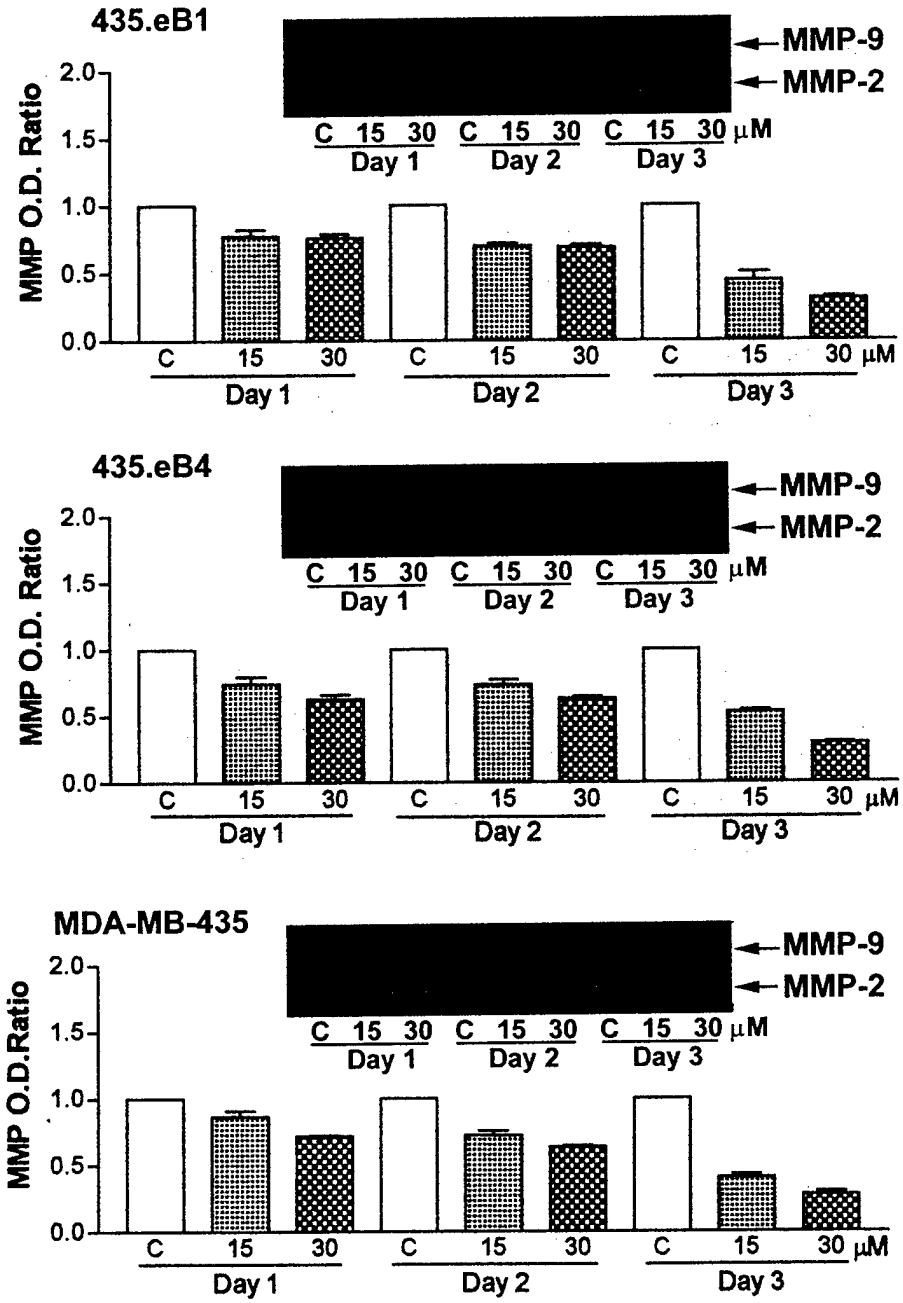

435.eB1
MMP-9
MMP-2
C 15 30 C 15 30 C 15 30 μM
Day 1 Day 2 Day 3
MMP O.D. Ratio
2.0
1.5
1.0
0.5
0.0
C 15 30 C 15 30 C 15 30 μM
Day 1 Day 2 Day 3
435.eB4
MMP-9
MMP-2
C 15 30 C 15 30 C 15 30 μM
Day 1 Day 2 Day 3
MMP O.D. Ratio
2.0
1.5
1.0
0.5
0.0
C 15 30 C 15 30 C 15 30 μM
Day 1 Day 2 Day 3
MDA-MB-435
MMP-9
MMP-2
C 15 30 C 15 30 C 15 30 μM
Day 1 Day 2 Day 3
MMP O.D.Ratio
2.0
1.5
1.0
0.5
0.0
C 15 30 C 15 30 C 15 30 μM
Day 1 Day 2 Day 3

Figure 4C