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The purpose of our investigation was to elucidate the molecular mechanism(s) by which genistein elicits its biological effects on non-tumorigenic and tumorigenic breast epithelial cells. We have completed all our tasks as planned and our major findings are: (a) that genistein inhibits cell cycle by modulating genes that are important cell cycle regulators, (b) that genistein induces apoptotic cell death in breast cancer cells irrespective of their status of estrogen receptor positivity, (c) that genistein elicits differential effects between non-tumorigenic and tumorigenic breast epithelial cells, (d) that genistein also down regulate NF-kB in breast epithelial cells and that this effect of genistein may be due to down regulation of Akt signaling (this aspect is currently being pursued in a pending grant application to the Department of Defense).

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## **INTRODUCTION:**

The subject of our study was to elucidate the molecular mechanism(s) by which soy metabolites, genistein and daidzein elicit their effects on breast epithelial cells. The purpose of our study was 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. To fully test our original hypothesis, we proposed three specific aims containing five tasks all of which has been successfully completed.

Since the submission of our previous annual progress report, we have completed the publication of a comprehensive article encompassing our latest observations and have completed some experiments toward the completion of our last objective. The last task was to dissect the molecular mechanism of genistein and daidzein on cell cycle regulatory proteins and apoptosis which has been accomplished. Our overall purpose of this study was to elucidate the molecular mechanism(s) by which the soy metabolite, genistein, exerts its biological effects on non-tumorigenic and tumorigenic breast epithelial cells. We were partly succeeded in accomplishing our overall goal during the tenure of the project, but to fully accomplish our goals, we obtained additional one year of no cost extension approval from the Department of Defense. Hence, this progress report should be considered as final report. The scope of our research was to establish whether there is any scientific basis to suggest that soy isoflavone, particularly genistein may have tumor cell specific biological effects in order to support the role of genistein in breast cancer prevention and/or treatment, and this scope has been fulfilled.

## **BODY:**

The following section describes the final progress to-date on our project. As stated in our last annual report that we have included several additional breast cancer cell lines than originally proposed in our grant application in order to improve our understanding on the role of genistein in breast cancer.

As indicated earlier that our objective was to further investigate the precise molecular mechanism(s) for the role of p21<sup>WAF1</sup> in our system. The results of our experiments was presented at the annual meeting of the American Association for Cancer Research (AACR) at San Francisco, and a comprehensive article has now been published (see attached article). These results are the final results on our project (see attached published article).

In summary, we have completed the project in full and 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 tumor cell specific irrespective of genetic differences. Furthermore, our data clearly demonstrate the differential effects of genistein on non-tumorigenic and tumorigenic breast epithelial cells, providing additional evidence for tumor cell specific effect of genistein. These results are original and demonstrate for the first time the molecular mechanism(s) by which genistein elicits its biological effects on human breast epithelial cells.

## **KEY RESEARCH ACCOMPLISHMENTS:**

- We have demonstrated a dose- and time-dependent cell growth inhibition and apoptosis in human breast cancer cells treated with genistein
- We have shown that genistein induces cell growth inhibition by inducing G2/M cell cycle arrest
- Our data clearly show alterations in some key genes that are important regulators of cell cycle arrest and apoptosis
- We have shown that genistein elicit its effects irrespective of the status of p53 and

- erbB-2 in breast cancer cells
- We have shown that genistein down-regulates erbB-2 and, in turn, down-regulates MMPs resulting in the inhibition of invasiveness and metastasis of breast cancer cells
- We have shown the differential effects of genistein in non-tumorigenic vs. tumorigenic breast epithelial cells
- Our data clearly show the role of p21<sup>WAF1</sup> in genistein-induced effects on breast epithelial cells
- Finally, we have documented a comprehensive molecular mechanism by which genistein elicits differential effects on normal vs. tumor cells, and that these molecular alterations are key events in inducing apoptosis induced by genistein in breast cancer cells.

#### **REPORTABLE OUTCOMES FOR THIS ENTIRE PERIOD:**

1. Li Y-W, Upadhyay S, Bhuiyan M, **Sarkar FH**. Induction of apoptosis in breast cancer cells MDA-MB-231 by genistein. *Oncogene* 18: 3166-3172, 1999.
2. Li Y-W, Bhuiyan M, **Sarkar FH**. Induction of apoptosis and inhibition of c-erbB-2 in MDA-MB-435 cells by genistein. *Int. J. Oncol.* 15: 525-533, 1999.
3. Upadhyay S, Neburi M, Chinni SR, Alhasan S, Miller F, **Sarkar FH**. Differential sensitivity of normal and malignant breast epithelial cells to genistein is partly mediated by p21(WAF1). *Clin. Cancer Res.* 7: 1782-1789, 2001.
4. Upadhyay S, **Sarkar FH**. Genistein induced growth inhibition but not apoptosis in untransformed MCF10A breast epithelial cells. *Proceedings of 89th annual meeting, AACR.* 39: Abstract 376, page-55, 1998.
5. Upadhyay S, Neburi M, **Sarkar FH**. Role of P21WAF1 in differential response to genistein in MCF10A derived cells. *Proceedings of 91st annual meeting, AACR* 41: A5365, page 845, 2000.
6. Sarkar FH, Upadhyay S. Induction of apoptosis in breast cancer cells by genistein. *Proceedings of the Department of Defense Breast Cancer Research Program Meeting "Era of Hope" Vol. II, page 463, 2000.*

#### **CONCLUSIONS:**

Collectively, we have accomplished all tasks completely as indicated under the statement of work. In addition, the results reported in the last manuscript (see attached published article) provided convincing evidence for the differential effects of genistein on non-tumorigenic vs. tumorigenic breast epithelial cells. Moreover, our data also show the molecular regulation and the role of p21<sup>WAF1</sup> in mediating the biological effects of genistein on breast epithelial cells. Overall, our results are important because, for the first time, we are providing convincing molecular evidence supporting that genistein may be an important agent for the prevention and/or treatment of breast cancer without any significant toxic effects on normal breast epithelium.

#### **APPENDICES:**

A reprint of last published paper.

## Differential Sensitivity of Normal and Malignant Breast Epithelial Cells to Genistein Is Partly Mediated by p21<sup>WAF1</sup>

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### ABSTRACT

Genistein, a soy metabolite, is a potential chemopreventive agent against various types of cancer. There are several studies documenting molecular alterations leading to cell cycle arrest and induction of apoptosis in a variety of cancer cells; however, no studies, to date, have shown the effect of genistein in isogenic normal and malignant breast epithelial cells. In this study, we investigated whether genistein shows any differential sensitivity to normal (MCF10A and MCF12A) and malignant (MCF10CA1a and MDA-MB-231) breast epithelial cells. We found that genistein causes a greater degree of G<sub>2</sub>-M arrest and induces apoptosis in malignant cell lines compared with normal breast epithelial cells. After genistein treatment, flow cytometric analysis revealed a hyperdiploid population in malignant cells that was not observed in normal cells. Cell cycle regulator p21<sup>WAF1</sup>, which is known to be up-regulated by genistein treatment, was greatly induced at RNA and protein levels in normal cells, whereas its level was only slightly induced in malignant MDA-MB-231 cells and not detectable in malignant MCF10CA1a cells. Therefore, we investigated the causal role of p21<sup>WAF1</sup> in the differential sensitivity of genistein among these cell lines.

We examined the effects of genistein on p21<sup>WAF1</sup> -/- and p21<sup>WAF1</sup> +/+ HCT116 cells, which were used as controls prior to studies on breast cancer cells. We found that there was a greater degree of cell cycle arrest and apoptosis in p21<sup>WAF1</sup> -/- cells compared with p21<sup>WAF1</sup> +/+ HCT116 cells after genistein treatment. Flow cytometric analysis after genistein treatment showed a significant number of p21<sup>WAF1</sup> -/- cells in the hyperdiploid population, which are probably programmed to die through apoptotic pro-

cesses. To further confirm the causal role of p21<sup>WAF1</sup> in genistein-mediated cell cycle arrest and apoptosis, we down-regulated p21<sup>WAF1</sup> by antisense p21<sup>WAF1</sup> cDNA transfection experiments. We found that both normal and malignant p21<sup>WAF1</sup> antisense (AS)-expressing clones became more sensitive to G<sub>2</sub>-M arrest after genistein treatment. Flow cytometric analysis showed an increase in the hyperdiploid population in the AS clones. Further evaluation showed an increase in apoptosis in malignant AS clones but not in normal breast epithelial AS clones. These results suggest that p21<sup>WAF1</sup> may play an important role in determining the sensitivity of normal and malignant breast epithelial cells to genistein.

### INTRODUCTION

The soy metabolite, phytochemical genistein, has been implicated as the anticancer component of the soy diet. Possible mechanisms for the antiproliferative property of genistein include: prevention of DNA mutation, reduction in cancer cell proliferation, inhibition of angiogenesis, and induction of differentiation (1). Epidemiological studies with Asian immigrants in the United States further suggest that susceptibility to breast cancer is partly attributable to environmental differences (especially diet) rather than genetic differences (2). There is an association between decreased breast cancer risk and increased phytochemical consumption (3, 4). Women who consume soy milk regularly have reduced levels of endogenous ovarian and adrenal hormones, which are recognized risk factors for breast cancer (5). More direct evidence linking phytochemicals to cancer prevention was observed in animal studies. Rats on a soy diet are protected from mammary tumor growth and progression (6, 7).

Genistein has been shown to inhibit cell proliferation of various cancer cell lines *in vitro* including both estrogen receptor-positive and estrogen receptor-negative breast carcinoma cell lines (8). It is generally accepted that genistein can arrest the cells at G<sub>2</sub>-M phase of the cell cycle (9), but a recent report has shown that genistein can also cause G<sub>0</sub>-G<sub>1</sub> arrest in a mouse fibroblast cell line (10). Collectively, these reports suggest that cell cycle arrest caused by genistein may be attributable to both G<sub>0</sub>-G<sub>1</sub> and G<sub>2</sub>-M arrest, depending on the cell lines and experimental conditions.

Antiproliferative actions of chemopreventive agents, including genistein, may be mediated by up-regulation of p21<sup>WAF1</sup> (11, 12). p21<sup>WAF1</sup> expression is usually controlled at the transcriptional level by both p53-dependent and p53-independent mechanisms. Introduction of p21<sup>WAF1</sup> expression constructs into normal (13) and tumor (14) cell lines results in cell cycle arrest in G<sub>1</sub> (15). p21<sup>WAF1</sup> appears to be solely responsible for G<sub>1</sub> arrest in human colon carcinoma cell line HCT116, because homozygous deletion of p21<sup>WAF1</sup> completely abrogates the G<sub>1</sub> checkpoint (16) and leads to a repair defect (17) after

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$\gamma$ -irradiation of these cells. Recently, it has been shown that p21<sup>WAF1</sup> may also mediate G<sub>2</sub> arrest (18, 19). p21<sup>WAF1</sup> may also have a role in apoptosis. Cells lacking p21<sup>WAF1</sup> appear to undergo apoptosis normally (20, 21). In other systems, transfection of p21<sup>WAF1</sup> has been found to protect cells from apoptosis (22, 23). Inactivation of p21<sup>WAF1</sup> sensitizes colorectal cancer cells to apoptosis by p53 (23). These results suggest that p21<sup>WAF1</sup> may play an important role in mediating apoptotic processes in genistein-treated breast cancer cells.

However, there have been no studies documenting the differential sensitivity of genistein in isogenic normal and malignant breast epithelial cells. Hence, the purpose of this study was to examine whether normal and malignant breast epithelial cells are differentially sensitive to genistein and to investigate whether p21<sup>WAF1</sup> plays any role in determining such differences in genistein sensitivity.

## MATERIALS AND METHODS

**Cell Lines.** For these studies, the following cell lines were used: MCF10A, MCF12A, MCF10CA1a (contains wild-type p53), MDA-MB-231 (contains mutant p53), HCT116, 80S14, and 379.2. MCF10A is spontaneously immortalized human breast epithelial cell line that was derived without viral or chemical intervention from mortal diploid human breast epithelial cells (24). The characteristics of this cell line and tissue culture condition are well established (25). MCF12A was derived from mortal diploid cells obtained from different patients than the MCF10A donor (26). MCF10A and MCF12A cells contain the wild-type p53 gene. MCF10CA1a cells were derived from MCF10A/MCF10NeoT model system (27);<sup>3</sup> hence, MCF10A and MCF10CA1a are considered isogenic. HCT116, 80S14, and 379.2 are colon cancer cell lines that were obtained from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). 80S14 and 379.2 cell lines are derived from HCT116. In 80S14 and 379.2, p21<sup>WAF1</sup> and p53 had been knocked out by homologous recombination, respectively.

**Cell Culture.** All cells were cultured in 95% air, 5% CO<sub>2</sub> at 37°C. MCF10A and MCF12A cells were cultured in DMEM/F-12 (1:1) medium (Life Technologies, Inc.) supplemented with 5% horse serum (Life Technologies, Inc.), 2 mM L-glutamine, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 1  $\mu$ g/ml insulin, 0.1  $\mu$ g/ml cholera toxin, 0.5  $\mu$ g/ml hydrocortisone (Sigma), 0.5  $\mu$ g/ml Fungizone, and 0.02  $\mu$ g/ml epidermal growth factor (Life Technologies, Inc.).

MCF10CA1a cells were cultured in DMEM/F-12 (1:1) medium supplemented with 5% horse serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. MCF10A, MCF12A, and MCF10CA1a cells were obtained from Karmanos Cancer Institute. MDA-MB-231 cells were cultured in DMEM/F-12 (1:1) medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 2 mM L-glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. HCT116,

80S14, and 379.2 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 2 mM L-glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin.

HN4 cells, derived from head and neck tumors at our institution, were grown in DMEM/F-12 (3:1) medium supplemented with 10% FBS, 0.2 mM adenine, 0.4  $\mu$ g/ml hydrocortisone, 0.1  $\mu$ g/ml cholera toxin, 5  $\mu$ g/ml transferrin, 5  $\mu$ g/ml insulin, and 2  $\times 10^{-11}$  triiodothyronine. HTB9 bladder cancer cells were grown in DMEM (high glucose) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 2 mM L-glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. H460 and H322 lung cancer cells were grown in RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin.

**Antibodies.** Mouse antihuman p21<sup>WAF1</sup> antibody was purchased from PharMingen (Lexington, KY). Mouse anti-PARP monoclonal antibody was purchased from Biomol, Inc. (Plymouth Meeting, PA).

**Flow Cytometry.** Cells were seeded at a density of 1  $\times 10^5$ /well in six-well culture dishes. The cells were treated with various concentrations of genistein for 3 days and harvested by trypsinization. The cells were centrifuged at 2000 rpm for 5 min, washed with PBS, and then fixed with 70% ethanol for at least 4 h. After fixation, cells were centrifuged at 2000 rpm, washed with PBS, and centrifuged again. Cell pellets were suspended in 1 ml of PBS with 0.1% Triton X-100 + 200  $\mu$ g/ml RNase + 200  $\mu$ g/ml propidium iodide for at least 1 h. Flow cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using the LYSYS II acquisition software package. The propidium iodide signal was detected by the FL-2 photomultiplier tube.

**Protein Extraction and Western Blot Analysis.** Whole cell lysates from control and genistein-treated cells were prepared using 2% SDS cell lysis buffer [2% SDS, 125 mM Tris-HCl (pH 6.8), and 20% glycerol]. Protein concentrations were measured using a commercial protein assay reagent (Pierce, Rockford, IL) to ensure equal loading. Twenty  $\mu$ g of proteins from whole cell lysates were mixed 1:1 with 2 $\times$  sample buffer and then applied to 10–14% polyacrylamide gels. Samples were electrophoretically separated and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membranes were incubated with specific primary antibodies and then incubated with antimouse or antirabbit peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA). After the incubation, the membranes were washed three times for 15 min each with 1 $\times$  TTBS solution and then incubated with 2 ml of chemiluminescence reagent (Pierce). The protein bands were visualized using X-ray films (Eastman Kodak, Rochester, NY).

**Northern Blot Analysis.** Total cellular RNA was isolated using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's protocol. RNA (10  $\mu$ g) from MCF10A, MCF10CA1a, and MDA-MB-231 cells were denatured and loaded on a formaldehyde-agarose (1%) gel. The RNA was transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH) using the Turbo-blotter (Schleicher & Schuell) in 20 $\times$  SSC buffer and subsequently UV cross-linked in a Stratagene (Stratagene, La Jolla, CA). The membrane was prehybridized and hybridized in 7 ml of hybridization buffer (0.25 M

<sup>3</sup> S. J. Santner, P. J. Dawson, L. Tait, H. D. Soule, J. Eliason, A. N. Mohamed, S. R., Wolman, G. H. Heppner, and F. R. Miller. Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells, submitted for publication.

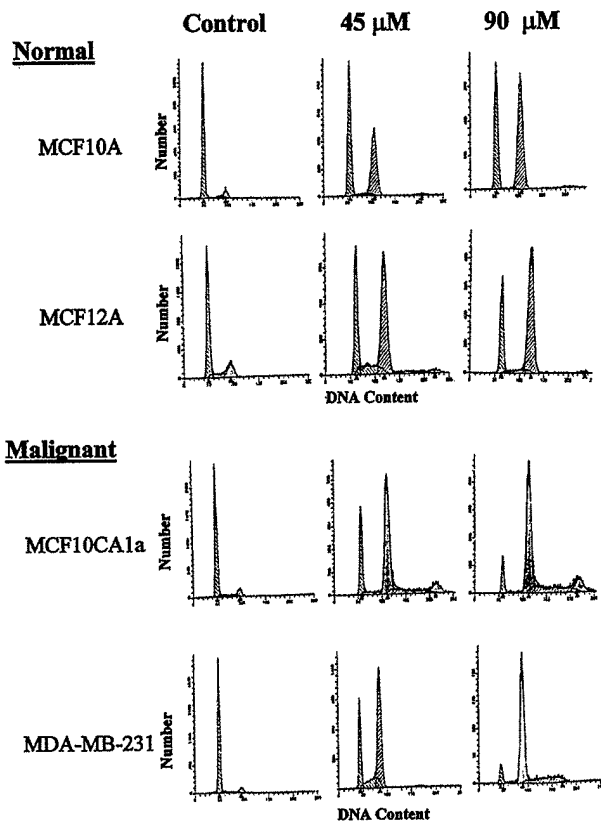


Fig. 1 Differential cell cycle effects of genistein between normal and malignant cells. Flow cytometric evaluation of cell cycle arrest in control and genistein-treated cells after 72 h. Numbers represent the percentage of cells in each phase of the cell cycle.

$\text{Na}_2\text{HPO}_4$  + 7% SDS) and  $^{32}\text{P}$ -labeled p21<sup>WAF1</sup> probe overnight at 65°C in hybridization buffer at constant rotation. After hybridization, the membrane was then washed twice (30 min each) in 20 mM  $\text{Na}_2\text{HPO}_4$  and 5% SDS at 65°C and again washed twice in 20 mM  $\text{Na}_2\text{HPO}_4$  and 1% SDS at 65°C. After washing, the membrane was wrapped in plastic paper, and the radioactive bands were detected using X-ray films.

**Gene Transfection Studies.** MCF10A and MDA-MB-231 cells were transfected with p21<sup>WAF1</sup> AS<sup>4</sup> cDNA, a gift from Dr. Vogelstein (Johns Hopkins University). The cDNA is under the control of the cytomegalovirus promoter and was introduced into these cells using FuGENE 6 (Boehringer Mannheim) reagent. Cells were plated in 100-mm dishes at a density of  $5 \times 10^5$ /dish. The next day, each culture dish was washed with PBS solution and overlaid with serum-free DMEM/F-12 medium. In a small sterile tube, 100  $\mu\text{l}$  of DMEM/F-12, 3  $\mu\text{l}$  of FuGENE 6, and 3  $\mu\text{g}$  of DNA were mixed and incubated for 20 min. Then it was transferred into the dishes containing serum-free medium and allowed to react with the cells overnight. On the next day, cells were replenished with complete medium and allowed to

Table 1 Flow cytometric cell cycle analysis of various normal and malignant cells

	G <sub>0</sub> -G <sub>1</sub>	S	G <sub>2</sub> -M	Hyperdiploid
<b>Nontumorigenic cell lines</b>				
<b>MCF10A</b>				
Control	84	8	8	0
45 $\mu\text{M}$	50	5	45	0
90 $\mu\text{M}$	40	2	58	0
<b>MCF12A</b>				
Control	70	15	15	0
45 $\mu\text{M}$	31	16	53	0
90 $\mu\text{M}$	28	7	65	0
<b>Tumorigenic cells</b>				
<b>MDA-MB-231</b>				
Control	87	7*	6	0
45 $\mu\text{M}$	24	16	58	2
90 $\mu\text{M}$	7	1	67	25
<b>MCF10ACA1a</b>				
Control	83	9	8	0
45 $\mu\text{M}$	20	2	53	25
90 $\mu\text{M}$	8	1	56	35
<b>HN4</b>				
Control	60	15	22	3
45 $\mu\text{M}$	14	22	52	12
90 $\mu\text{M}$	6	16	31	47
<b>HTB9</b>				
Control	75	19	5	0
45 $\mu\text{M}$	29	6	60	5
90 $\mu\text{M}$	14	4	70	12
<b>H460</b>				
Control	71	16	13	0
45 $\mu\text{M}$	26	20	49	5
90 $\mu\text{M}$	15	34	35	16

recover for 48 h. After 48 h, hygromycin selection was started with the following concentrations: 100 and 700  $\mu\text{g}/\text{ml}$  for MCF10A and MDA-MB-231 cells, respectively. Eighteen clones for each cell line were obtained and used for additional studies.

**Luciferase Assays.** Five  $\times 10^5$  cells/well were plated 24 h prior to transfection in six-well plates. The next day, luciferase DNA constructs and  $\beta$ -galactosidase expression plasmid pCH100 were transfected per well for 24 h in serum-free medium, using FuGENE 6 (Boehringer Mannheim). On the next day, complete medium was added, and 24 h later, cells were treated with genistein for 24 h. Cells were lysed in reporter lysis buffer (Promega Corp., Madison, WI), and lysates were assayed for luciferase activity using a luminometer. Lysates were also assayed for  $\beta$ -galactosidase activity to normalize for transfection efficiency.

## RESULTS

**Genistein Induces Cell Cycle Arrest.** To determine whether isogenic normal and malignant breast epithelial cells are differentially sensitive to genistein, we chose immortalized normal breast epithelial cell lines MCF10A and MCF12A and malignant cell lines MCF10CA1a and MDA-MB-231. These cells were treated with various concentrations of genistein and evaluated for cell cycle arrest by flow cytometric analysis. Genistein is known to cause G<sub>2</sub>-M cell cycle arrest in many different cancer cell lines. Treatment with genistein resulted in G<sub>2</sub>-M cell cycle arrest in all cells tested. However, this effect

<sup>4</sup> The abbreviations used are: AS, antisense; PARP, poly(ADP-ribose) polymerase.

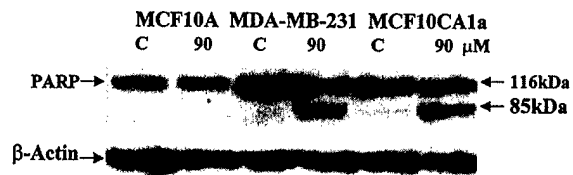


Fig. 2 Genistein induces differential apoptotic response between normal and malignant cells. Western blot analysis of PARP cleavage of control and genistein-treated cells after 72 h.

was much more pronounced in malignant cells compared with normal cells (Fig. 1). Interestingly, there was an appearance of a hyperdiploid population in malignant cells, after genistein treatment, which was not observed in normal cells (Fig. 1). In normal cells, there were 58% (MCF10A) and 62% (MCF12A) cells in the G<sub>2</sub>-M phase of the cell cycle after 90 μM genistein treatment. The rationale for using high concentrations of genistein such as 45 and 90 μM was based on our preliminary studies, which showed some effect of genistein on normal breast epithelial cells. In contrast, low concentrations of genistein such as 5–30 μM did not show any activity on normal breast epithelial cells but did show significant antiproliferative activity in malignant cells. Therefore, to compare molecular effects of genistein in both normal and malignant cells, high concentration was considered for our experiments. Although G<sub>2</sub>-M cell cycle arrest was observed, a hyperdiploid population was not detected in normal cells. On the other hand, in malignant cells, there were 91% (MCF10CA1a) and 92% (MDA-MB-231) of cells in G<sub>2</sub>-M and hyperdiploid phases after 90 μM genistein treatment (Table 1). We have reported previously a similar effect of genistein in head and neck and lung cancer cell lines, respectively (28, 29) as summarized in Table 1. These malignant cell lines also exhibited hyperdiploid populations after genistein treatment (Table 1).

**Genistein Induces Apoptosis in Malignant Breast Epithelial Cells.** Genistein is known to induce apoptosis in many different cancer cells lines (9, 28–30). A cascade of events, whereby proteases such as caspase-3 are cleaved, marks apoptotic processes. This activated caspase-3 then cleaves substrates such as PARP. Cleavage of PARP, a M<sub>r</sub> 116,000 molecular weight protein, during apoptosis results in a M<sub>r</sub> 85,000 product, which can be visualized by Western blot analysis. To determine whether there is any differential apoptotic response of genistein in normal and malignant cells, PARP cleavage after genistein treatment was examined. Three days after 90 μM genistein treatment, an apoptotic cleavage fragment of PARP was readily detected in malignant MDA-MB-231 and MCF10CA1a cells but not in normal MCF10A (Fig. 2) and MCF12A (data not shown) cells. These results suggest that malignant cells are more sensitive to genistein-mediated cell cycle arrest and apoptosis compared with normal breast epithelial cells.

**Genistein Effects on p21<sup>WAF1</sup> Expression.** p21<sup>WAF1</sup> has been shown to be modulated by genistein. p21<sup>WAF1</sup> has also been shown to play an important role in G<sub>2</sub>-M cell cycle arrest and apoptosis. To determine whether p21<sup>WAF1</sup> may be differentially modulated in normal and malignant cells after genistein treatment, the levels of p21<sup>WAF1</sup> in control and genistein-treated

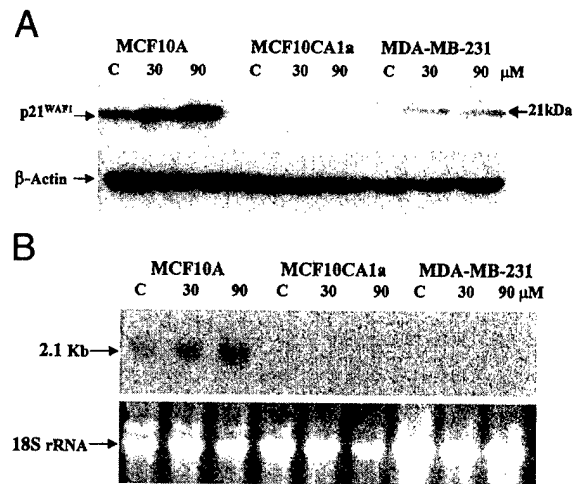


Fig. 3 A, differential induction of p21<sup>WAF1</sup> protein by genistein in normal and malignant cells. Western blot analysis of control and genistein-treated cells after 72 h. B, differential induction of p21<sup>WAF1</sup> mRNA by genistein in normal and malignant cells. Northern blot analysis of total RNA from control and genistein-treated cells after 72 h.

Table 2 Average luciferase activity (mean ± SD) in nontumorigenic and tumorigenic control and genistein-treated cells

p21(+) refers to wild-type promoter and p21(-) refers to mutant promoter without the p53 binding site.

	Luciferase activity
MCF10A	
p21(-) untreated	334 ± 52
p21(-) treated	704 ± 95
MCF10A	
p21(+) untreated	1377 ± 82
p21(+) treated	5448 ± 413
MCF10CA1a	
p21(+) untreated	79 ± 4.5
p21(+) treated	110 ± 7.5
MDA-MB-231	
p21(+) untreated	45 ± 7
p21(+) treated	97 ± 5

MCF10A, MCF10CA1a, and MDA-MB-231 cells were examined. Three days after genistein treatment, p21<sup>WAF1</sup> protein levels increased 4–5-fold in genistein-treated MCF10A cells, compared with control cells (Fig. 3A). There was only a slight increase in p21<sup>WAF1</sup> levels in genistein-treated MDA-MB-231 cells, whereas no p21<sup>WAF1</sup> was detected in MCF10CA1a cells (Fig. 3A). These results suggest that genistein treatment of normal MCF10A cells results in a more pronounced induction of p21<sup>WAF1</sup> levels compared with malignant MDA-MB-231 and MCF10CA1a cells. We have shown that genistein modulates p21<sup>WAF1</sup> to a greater extent in MCF10A cells compared with MDA-MB-231 and MCF10CA1a cells. Modulation of p21<sup>WAF1</sup> by genistein could be attributable to changes in transcriptional activity of the p21<sup>WAF1</sup> promoter. Thus, the p21<sup>WAF1</sup> promoter might be differentially modulated by genistein in MCF10A, MCF10CA1a, and MDA-MB-231 cells, leading to differential modulation of p21<sup>WAF1</sup> protein in these cells.

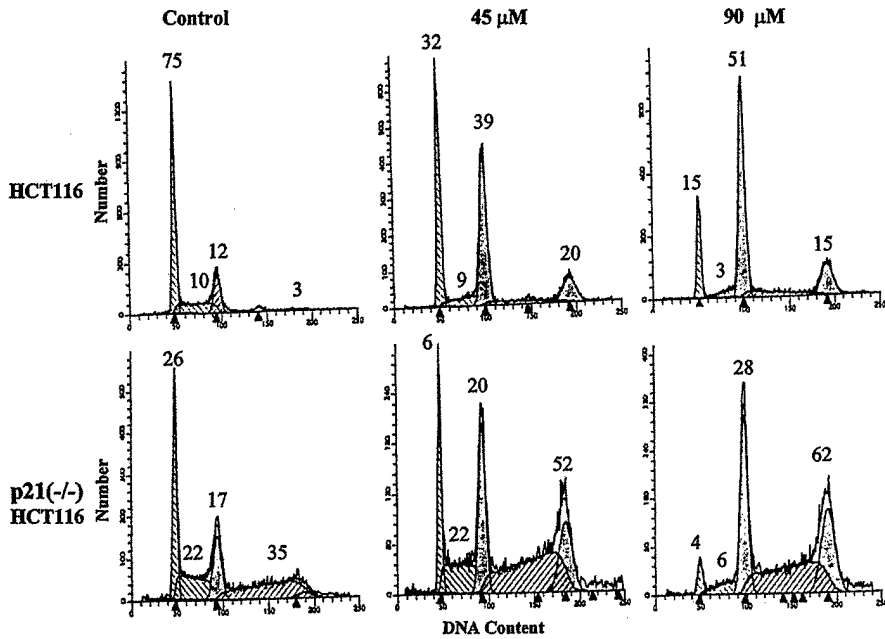


Fig. 4 Genistein induces a differential cell cycle effect in p21<sup>WAF1</sup> -/- and parental HCT116 cells. Flow cytometric evaluation of cell cycle arrest in control and genistein-treated cells after 72 h is shown, as is cell cycle distribution of HCT116 and p21 -/- cells after genistein treatment. Numbers represent the percentage of cells in each phase of the cell cycle.

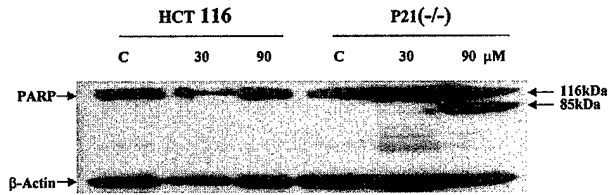


Fig. 5 Genistein induces differential apoptotic response in p21<sup>WAF1</sup> -/- and parental HCT116 cells. Western blot analysis of PARP cleavage in control and genistein-treated cells after 72 h is shown.

**Genistein Effects on p21<sup>WAF1</sup> Promoter Activity.** To determine the differential activation of the p21<sup>WAF1</sup> promoter, we first examined p21<sup>WAF1</sup> RNA levels in control and genistein-treated MCF10A, MCF10CA1a, and MDA-MB-231 cells, using Northern blot analysis. Genistein treatment resulted in a 3–4-fold increase in p21<sup>WAF1</sup> mRNA in MCF10A cells, whereas there was a slight increase in p21<sup>WAF1</sup> observed in MDA-MB-231 cells. We were unable to detect p21<sup>WAF1</sup> RNA in either control or genistein-treated MCF10CA1a cells (Fig. 3B). Because promoter activation can lead to changes in RNA levels, we performed the luciferase assay in control and genistein-treated MCF10A, MCF10CA1a, and MDA-MB-231 cells, using the p21<sup>WAF1</sup> promoter. p53 is known to activate the p21<sup>WAF1</sup> promoter. To determine whether activation of the p21<sup>WAF1</sup> promoter by genistein is p53 dependent, we transfected the p21<sup>WAF1</sup> promoter without the p53 binding site, designated as p21(-), into MCF10A cells and performed luciferase assays. Genistein treatment resulted in only a 2-fold activation of the p21<sup>WAF1</sup> promoter. On the other hand, genistein treatment after transfection of the wild-type promoter, designated as p21(+), resulted in a 4-fold activation of p21<sup>WAF1</sup> promoter activity in MCF10A cells. Also, the basal promoter

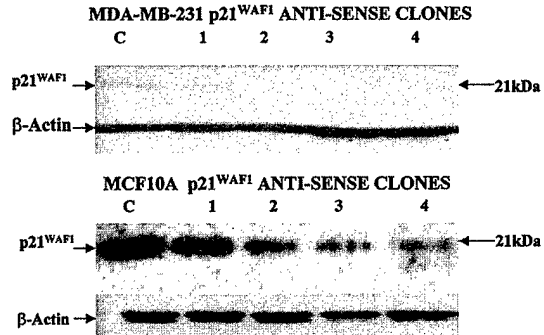


Fig. 6 Western blot analysis of p21<sup>WAF1</sup> protein from cell lysates of control, MDA-MB-231 p21<sup>WAF1</sup> AS clones, and MCF10A p21<sup>WAF1</sup> AS clones.

activity of wild-type p21<sup>WAF1</sup> promoter was much greater compared with the p21<sup>WAF1</sup> promoter without the p53 binding site. Only a modest activation of p21<sup>WAF1</sup> promoter activity was observed in MCF10CA1a and MDA-MB-231 cells (Table 2). In summary, differential G<sub>2</sub>-M cell cycle arrest and apoptosis between normal and malignant cells appear to be mediated by a differential effect of genistein on p21<sup>WAF1</sup>. It appears that this process may be p53 dependent, which is the subject of our future investigation.

**Lack of p21<sup>WAF1</sup> Contributes to Genistein-mediated Apoptosis and Cell Cycle Arrest.** To confirm the role of p21<sup>WAF1</sup> in genistein-mediated cell cycle arrest and apoptosis, we used the p21<sup>WAF1</sup> knockout (-/-) colon cancer cell line 80S14 as a control, in which both copies of the p21<sup>WAF1</sup> were knocked out by homologous recombination, and the results were compared with the parental HCT116 cells. To determine whether a lack of p21<sup>WAF1</sup> sensitizes cells to cell cycle arrest,

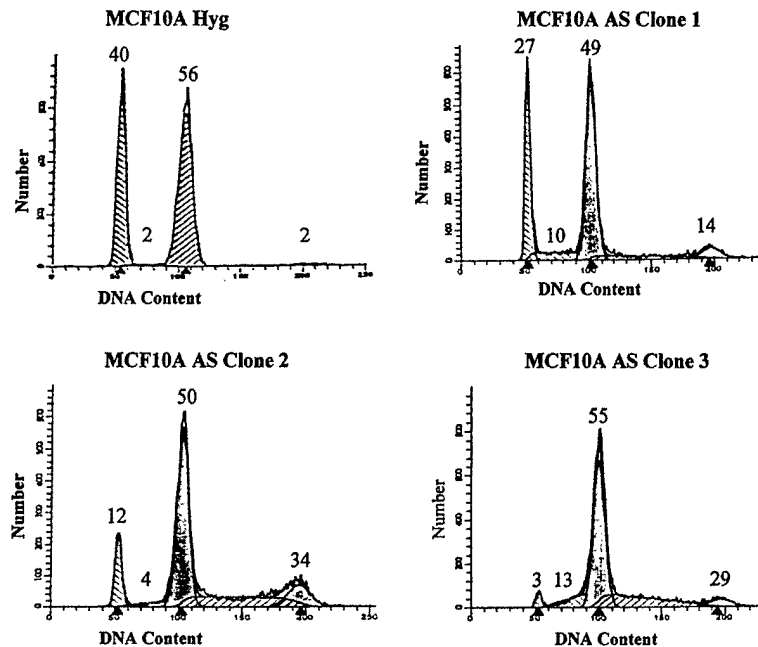


Fig. 7 Increased cell cycle arrest of MCF10A p21<sup>WAF1</sup> AS clones compared with control cells. Flow cytometric analysis of cell cycle distribution of MCF10A Hyg and p21<sup>WAF1</sup> AS clones after 90  $\mu$ M genistein treatment for 72 h is shown. Numbers represent the percentage of cells in each phase of the cell cycle.

we performed flow cytometric analysis on genistein-treated p21<sup>WAF1</sup>  $-/-$  and p21<sup>WAF1</sup>  $+/+$  HCT116 cells. We found that in both cell lines, there was an increase in G<sub>2</sub>-M cell cycle arrest (Fig. 4). However, there were important differences observed. After 90  $\mu$ M genistein treatment, p21<sup>WAF1</sup>  $-/-$  cells showed 4-fold fewer cells in G<sub>0</sub>-G<sub>1</sub>, compared with parental HCT116 cells (Fig. 4). Furthermore, genistein-treated p21<sup>WAF1</sup>  $-/-$  cells showed a 4-fold increase in the hyperdiploid population compared with HCT116 cells (Fig. 4). After 90  $\mu$ M genistein treatment, there were ~25% more cells in the G<sub>2</sub>-M and hyperdiploid phases of the cell cycle in p21<sup>WAF1</sup>  $-/-$  cells compared with parental HCT116 cells. These results suggest that p21<sup>WAF1</sup> is important for genistein mediated G<sub>2</sub>-M arrest and that the lack of p21<sup>WAF1</sup> sensitizes cells to genistein-mediated cell cycle arrest.

**Down-Regulation of p21<sup>WAF1</sup> Contributes to Genistein-mediated Apoptosis and Cell Cycle Arrest.** To determine the role of p21<sup>WAF1</sup> in genistein-mediated apoptosis, we investigated PARP cleavage. Three days after genistein treatment, the level of  $M_r$  85,000 apoptotic cleavage fragment of PARP was much greater in p21<sup>WAF1</sup>  $-/-$  cells compared with parental cells (Fig. 5). Visual observation did show some cell death in p21<sup>WAF1</sup>  $+/+$  cells. Therefore, lack of p21<sup>WAF1</sup> appears to sensitize these cells to genistein-mediated apoptosis.

To further confirm the role of p21<sup>WAF1</sup> in sensitizing cells to genistein-mediated effects, we down-regulated p21<sup>WAF1</sup> by transfecting AS p21<sup>WAF1</sup> cDNA. Previously, we showed that p21<sup>WAF1</sup> is up-regulated in MCF10A and MDA-MB-231 cells after genistein treatment. Hence, we transfected these cells with the p21<sup>WAF1</sup> AS cDNA expression vector under the control of the cytomegalovirus promoter and a hygromycin-resistant control vector. After hygromycin selection, we obtained 18 p21<sup>WAF1</sup> AS clones of MCF10A and MDA-MB-231 cells. p21<sup>WAF1</sup> expression levels were determined by Western blot

analysis (Fig. 6) in these clones. In MCF10A AS cells, p21<sup>WAF1</sup> expression was reduced 50–67% compared with parental cells. In MDA-MB-231 AS cells, p21<sup>WAF1</sup> expression was reduced 50–80% compared with parental cells. To determine whether p21<sup>WAF1</sup> AS clones are more sensitive to cell cycle arrest by genistein, we performed flow cytometric analysis on AS clones after genistein treatment. In MCF10A cells, we had shown previously that 90  $\mu$ M genistein caused 58% of the cells to arrest in the G<sub>2</sub>-M phase of the cell cycle. Flow cytometric analysis showed that 84% of MCF10A p21<sup>WAF1</sup> AS clone 3 in the G<sub>2</sub>-M and hyperdiploid phases of the cell cycle, after 90  $\mu$ M genistein treatment (Fig. 7). Cells with greater p21<sup>WAF1</sup> down-regulation had increased proportion of cells arrested at G<sub>2</sub>-M and a larger hyperdiploid population (Fig. 7). Similar results were obtained from MDA-MB-231 p21<sup>WAF1</sup> AS clones. Genistein treatment (45  $\mu$ M) of MDA-MB-231 cells caused 60% of the cells to arrest in the G<sub>2</sub>-M and hyperdiploid phases compared with 80% of MDA-MB-231 p21<sup>WAF1</sup> AS clone 2 after 45  $\mu$ M genistein (Fig. 8). MDA-MB-231 AS clones with greater p21<sup>WAF1</sup> down-regulation showed greater growth arrest and hyperdiploid population (Fig. 8).

Because p21<sup>WAF1</sup>  $-/-$  colon carcinoma cells were more sensitive to apoptotic cell death compared with p21<sup>WAF1</sup>  $+/+$  HCT116 cells after genistein treatment, we investigated whether a decrease in p21<sup>WAF1</sup> in the MCF10A normal breast and MDA-MB-231 malignant breast cells could also enhance sensitivity to genistein-mediated apoptotic cell death. We treated MCF10A and MDA-MB-231 p21<sup>WAF1</sup> AS clones with 90  $\mu$ M genistein and examined PARP cleavage. Three days of genistein treatment did not induce apoptosis in MCF10A p21<sup>WAF1</sup> AS clones (Fig. 9). However, apoptosis-specific PARP cleavage was greatly enhanced in MDA-MB-231 p21<sup>WAF1</sup> AS clones compared with parental cells (Fig. 9). These results suggest that a partial down-regulation of p21<sup>WAF1</sup> in normal breast epithelial

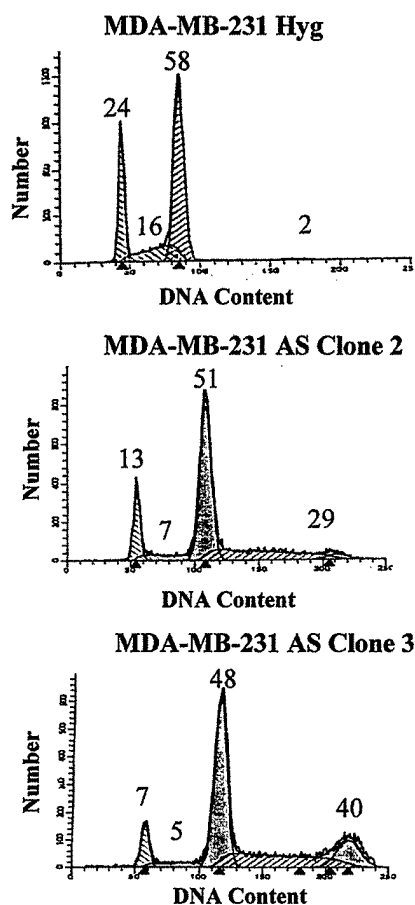


Fig. 8 Increased cell cycle arrest of MDA-MB-231 p21<sup>WAF1</sup> AS clones compared with control cells. Flow cytometric analysis of cell cycle distribution of MDA-MB-231 Hyg and p21<sup>WAF1</sup> AS clones after 45  $\mu$ M genistein treatment for 72 h. Numbers represent the percentage of cells in each phase of the cell cycle.

cells (MCF10A) was not sufficient to trigger apoptosis. However, cancer cells could be sensitized to enhanced killing by down-regulation of p21<sup>WAF1</sup>.

## DISCUSSION

The selective growth inhibition of Ha-Ras transformed NIH3T3 (31) cells by genistein was the first evidence that oncogenic transformation can sensitize cells to chemopreventive agents. Recently, another study using capsaicin, a phytochemical found in red pepper, showed selective growth inhibition of Ha-Ras transformed MCF10A cells.<sup>5</sup> These studies provided important clues as to whether certain genes and pathways may play an important role in determining the biological effects of chemopreventive agents. Furthermore, the oncogenic process may be fundamentally impor-

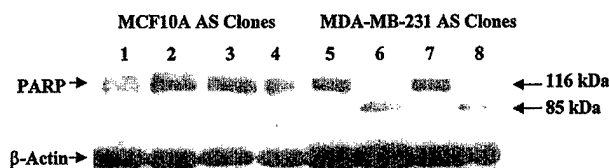


Fig. 9 Differential apoptotic response of MCF10A p21<sup>WAF1</sup> AS clones 2 and 3 and MDA-MB-231 p21<sup>WAF1</sup> AS clones 2 and 3 to genistein treatment. Western blot analysis of PARP cleavage in untreated (Lanes 1, 3, 5, and 7) and genistein-treated (Lanes 2, 4, 6, and 8) MCF10A and p21<sup>WAF1</sup> AS clones and MDA-MB-231 and p21<sup>WAF1</sup> AS clones, respectively, after 72 h is shown.

tant for increased susceptibility of cancer cells to chemopreventive agents compared with normal cells.

In the present study, we showed that p21<sup>WAF1</sup> plays an important role in eliciting differential sensitivity of isogenic normal and malignant breast epithelial cells to genistein. We have also shown that malignant cells are more sensitive to G<sub>2</sub>-M cell cycle arrest, hyperdiploid progression, and induction of apoptosis by genistein. Molecular profiling of these effects showed a greater induction of p21<sup>WAF1</sup> in normal cells compared with malignant cells. Therefore, we investigated whether p21<sup>WAF1</sup> plays any role in eliciting differential effects of genistein in normal and malignant cells.

We used p21<sup>WAF1</sup> <sup>-/-</sup> and p21<sup>WAF1</sup> <sup>+/+</sup> HCT116 cells to further investigate the role of p21<sup>WAF1</sup> in genistein-mediated effects. Our studies revealed that p21<sup>WAF1</sup> <sup>-/-</sup> cells were more sensitive to cell cycle arrest and induction of apoptosis compared with p21<sup>WAF1</sup> <sup>+/+</sup> cells. We further confirmed the role of p21<sup>WAF1</sup> in sensitizing cells to genistein by down-regulating p21<sup>WAF1</sup> with AS cDNA transfection experiments using breast MCF10A and MDA-MB-231 cells. Our cell cycle and apoptosis analysis with the AS clones supported the hypothesis that down-regulation of p21<sup>WAF1</sup> in cancer cells makes them more sensitive to genistein-mediated effects, compared with normal cells. Our p21<sup>WAF1</sup> luciferase assays and Northern blot analysis supported the contention that, indeed, there are differences in the level of promoter activity that could be the basis for differential induction of p21<sup>WAF1</sup> levels after genistein treatment.

Many studies have shown up-regulation of p21<sup>WAF1</sup> and subsequent apoptosis in various cancer cell lines by genistein (9, 30). Up-regulation of p21<sup>WAF1</sup> observed in these cell lines is, perhaps, attributable to a stress response and may not be directly related to genistein-induced apoptosis. MCF10A cells did not undergo apoptosis when exposed to high concentrations of genistein; yet these cells show up-regulation of p21<sup>WAF1</sup>. Therefore, we believe that up-regulation of p21<sup>WAF1</sup> prevents apoptosis and induces cell cycle arrest. Certainly, it is possible that a combination of genetic make-up and up-regulation of p21<sup>WAF1</sup> makes malignant cells more sensitive to apoptosis.

The presence of hyperdiploid DNA in malignant cells, but not in normal cells, suggests inappropriate cell cycle activity in cancer cell lines. Our experiments have correlated decreased p21<sup>WAF1</sup> levels with improper cell cycle activity and apoptosis after genistein treatment, raising the possibility that abnormal cell cycle response to genistein in malignant cells triggers an apoptotic response. One possible mechanism through which hyperdiploid

<sup>5</sup> H. Kang, Y. Soh, M. Kim, Y. Surh, H-R. Kim, and A. Moon. Capsaicin induces apoptosis in ras-transformed human breast epithelial cells through modulation of JNK and ERKs, submitted for publication.

progression may occur in malignant cells that contribute to cell death is via modulation of apoptosis regulators such as Bcl-2, Bax, and caspases, as reported previously (32, 33). In addition, hyperdiploid cells may be further sensitized to the effects of genistein because of lack of repair (17). These results are also supported by recent studies showing that cells lacking p21<sup>WAF1</sup> acquire polyploidy and ultimately die through apoptotic processes (34, 35). However, further in-depth investigations are needed that will establish whether genistein-induced hyperdiploidy is causally related to apoptotic processes in breast cancer cells. In summary, our data suggest that genistein may have wide applications because of its selective inhibition of malignant cells without any significant effect on normal cells for the treatment and/or prevention of breast cancer.

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## REFERENCES

1. Knight, D. C., and Eden, J. A. A review of the clinical effects of phytoestrogens. *Obstet. Gynecol.*, **87**: 897-904, 1996.
2. Committee on Diet, Nutrition, and Cancer, Assembly of Life Sciences, National Research Council. *Diet, Nutrition, and Cancer*. Washington, DC: National Academy Press, 1982.
3. Adlercreutz, H. Phytoestrogens: epidemiology and a possible role in cancer protection. *Environ. Health Perspect.*, **103** (Suppl 7): 103-112, 1995.
4. Lee, H. P., Gourley, L., Duffy, S. W., Esteve, J., Lee, J., and Day, N. E. Dietary effects on breast-cancer risk in Singapore. *Lancet*, **337**: 1197-1200, 1991.
5. Lu, L. J., Anderson, K. E., Grady, J. J., and Nagamani, M. Effects of soya consumption for one month on steroid hormones in premenopausal women: implications for breast cancer risk reduction. *Cancer Epidemiol. Biomark. Prev.*, **5**: 63-70, 1996.
6. Barnes, S., Grubbs, C., Setchell, K. D., and Carlson, J. Soybeans inhibit mammary tumors in models of breast cancer. *Prog. Clin. Biol. Res.*, **347**: 239-253, 1990.
7. Hawrylewicz, E. J., Huang, H. H., and Blair, W. H. Dietary soybean isolate and methionine supplementation affect mammary tumor progression in rats. *J. Nutr.*, **121**: 1693-1698, 1991.
8. Peterson, G., and Barnes, S. Genistein inhibition of the growth of human breast cancer cells: independence from estrogen receptors and the multidrug resistance gene. *Biochem. Biophys. Res. Commun.*, **179**: 661-667, 1991.
9. Pagliacci, M. C., Smacchia, M., Migliorati, G., Grignani, F., Riccardi, C., and Nicoletti, I. Growth-inhibitory effects of the natural phyto-estrogen genistein in MCF-7 human breast cancer cells. *Eur. J. Cancer*, **11**: 1675-1682, 1994.
10. Kuzumaki, T., Kobayashi, T., and Ishikawa, K. Genistein induces p21(Cip1/WAF1) expression and blocks the G<sub>1</sub> to S phase transition in mouse fibroblast and melanoma cells. *Biochem. Biophys. Res. Commun.*, **251**: 291-295, 1998.
11. Lallemand, F., Courilleau, D., Buquet-Fagot, C., Atfi, A., Montagne, M. N., and Mester, J. Sodium butyrate induces G<sub>2</sub> arrest in the human breast cancer cells MDA-MB-231 and renders them competent for DNA rereplication. *Exp. Cell Res.*, **247**: 432-440, 1999.
12. Lin, J. K., Liang, Y. C., and Lin-Shiau, S. Y. Cancer chemoprevention by tea polyphenols through mitotic signal transduction blockade. *Biochem. Pharmacol.*, **58**: 911-915, 1999.
13. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G<sub>1</sub> cyclin-dependent kinases. *Cell*, **75**: 805-816, 1993.
14. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**: 817-825, 1993.
15. Harper, J. W., Elledge, S. J., Keyomarsi, K., Dynlacht, B., Tsai, L. H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., Swindell, E., *et al.* Inhibition of cyclin-dependent kinases by p21. *Mol. Cell Biol.*, **6**: 387-400, 1995.
16. Waldman, T., Kinzler, K. W., and Vogelstein, B. p21 is necessary for the p53-mediated G<sub>1</sub> arrest in human cancer cells. *Cancer Res.*, **55**: 5187-5190, 1995.
17. McDonald, E. R., III, Wu, G. S., Waldman, T., and El-Deiry, W. S. Repair defect in p21<sup>WAF1/CIP1</sup> -/- human cancer cells. *Cancer Res.*, **56**: 2250-2255, 1996.
18. Dulic, V., Stein, G. H., Far, D. F., and Reed, S. I. Nuclear accumulation of p21Cip1 at the onset of mitosis: a role at the G<sub>2</sub>/M-phase transition. *Mol. Cell Biol.*, **18**: 546-557, 1998.
19. Niculescu, A. B., III, Chen, X., Smeets, M., Hengst, L., Prives, C., and Reed, S. I. Effects of p21(Cip1/Waf1) at both the G<sub>1</sub>/S and the G<sub>2</sub>/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. *Mol. Cell Biol.*, **18**: 629-643, 1998.
20. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leder, P. Mice lacking p21CIP1/WAF1 undergo normal development but are defective in G<sub>1</sub> checkpoint control. *Cell*, **82**: 675-684, 1995.
21. Brown, J. P., Wei, W., and Sedivy, J. M. Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts. *Science (Wash. DC)*, **277**: 831-834, 1997.
22. Gorospe, M., Cirielli, C., Wang, X., Seth, P., Capogrossi, M. C., and Holbrook, N. J. p21(Waf1/Cip1) protects against p53-mediated apoptosis of human melanoma cells. *Oncogene*, **14**: 929-935, 1997.
23. Polyak, K., Waldman, T., He, T. C., Kinzler, K. W., and Vogelstein, B. Genetic determinants of p53-induced apoptosis and growth arrest. *Genes Dev.*, **10**: 1945-1952, 1996.
24. Soule, H. D., Maloney, T. M., Wolman, S. R., Peterson, W. D., Jr., Brenz, R., McGrath, C. M., Russo, J., Pauley, R. J., Jones, R. F., and Brooks, S. C. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res.*, **50**: 6075-6086, 1990.
25. Tait, L., Soule, H. D., and Russo, J. Ultrastructural and immunocytochemical characterization of an immortalized human breast epithelial cell line, MCF-10. *Cancer Res.*, **50**: 6087-6094, 1990.
26. Wolman, S. R., Mohamed, A. N., Heppner, G. H., and Soule, H. D. Chromosomal markers of immortalization in human breast epithelium. *Genes Chromosomes Cancer*, **10**: 59-65, 1994.
27. Miller, F. R., Soule, H. D., Tait, L., Pauley, R. J., Wolman, S. R., Dawson, P. J., and Heppner, G. H. Xenograft model of progressive human proliferative breast disease. *J. Natl. Cancer Inst.*, **85**: 1725-1732, 1993.
28. Lian, F., Li, Y.-W., Bhuiyan, M., and Sarkar, F. H. p53 independent apoptosis induced by genistein in lung cancer cells. *Nutr. Cancer*, **33**: 125-131, 1999.
29. Alhasan, S. A., Ensley, J., and Sarkar, F. H. Genistein induced molecular changes in a squamous cell carcinoma of the head and neck cell line. *Int. J. Oncol.*, **16**: 333-338, 2000.
30. Shao, Z. M., Wu, J., Shen, Z. Z., and Barsky, S. H. Genistein exerts multiple suppressive effects on human breast carcinoma cells. *Cancer Res.*, **58**: 4851-4857, 1998.
31. Okura, A., Arakawa, H., Oka, H., Yoshinari, T., and Monden, Y. Effect of genistein on topoisomerase activity and on the growth of [Val 12]Ha-ras-transformed NIH 3T3 cells. *Biochem. Biophys. Res. Commun.*, **157**: 183-189, 1988.
32. Thornberry, N. A., and Lazebnik, Y. Caspases: enemies within. *Science (Wash. DC)*, **281**: 1312-1316, 1998.
33. Adams, J. M., and Cory, S. The Bcl-2 protein family: arbiters of cell survival. *Science (Wash. DC)*, **281**: 1322-1326, 1998.
34. Waldman, T., Lengauer, C., Kinzler, K. W., and Vogelstein, B. Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. *Nature (Lond.)*, **381**: 713-716, 1996.
35. Bulavin, D. V., Tararova, N. D., Aksenov, N. D., Pospelov, V. A., and Pospelova, T. V. Deregulation of p53/p21Cip1/Waf1 pathway contributes to polyploidy and apoptosis of E1A+cHa-ras transformed cells after  $\gamma$ -irradiation. *Oncogene*, **18**: 5611-5619, 1999.