Differential Sensitivity of Normal and Malignant Breast Epithelial Cells to Genistein Is Partly Mediated by $p21^{WAF1}$ [1]

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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_2-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 $p21^{WAF1}$, 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 $p21^{WAF1}$ in the differential sensitivity of genistein among these cell lines.

We examined the effects of genistein on $p21^{WAF1}$ −/− and $p21^{WAF1}$ +/+ 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 $p21^{WAF1}$ −/− cells compared with $p21^{WAF1}$ +/+ HCT116 cells after genistein treatment. Flow cytometric analysis after genistein treatment showed a significant number of $p21^{WAF1}$ −/− cells in the hyperdiploid population, which are probably programmed to die through apoptotic processes. To further confirm the causal role of $p21^{WAF1}$ in genistein-mediated cell cycle arrest and apoptosis, we down-regulated $p21^{WAF1}$ by antisense $p21^{WAF1}$ cDNA transfection experiments. We found that both normal and malignant $p21^{WAF1}$ antisense (AS)-expressing clones became more sensitive to G_2-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 $p21^{WAF1}$ 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_2-M phase of the cell cycle (9), but a recent report has shown that genistein can also cause G_0-G_1 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_0-G_1 and G_2-M arrest, depending on the cell lines and experimental conditions.

Antiproliferative actions of chemopreventive agents, including genistein, may be mediated by up-regulation of $p21^{WAF1}$ (11, 12). $p21^{WAF1}$ expression is usually controlled at the transcriptional level by both p53-dependent and p53-independent mechanisms. Introduction of $p21^{WAF1}$ expression constructs into normal (13) and tumor (14) cell lines results in cell cycle arrest in G_1 (15). $p21^{WAF1}$ appears to be solely responsible for G_1 arrest in human colon carcinoma cell line HCT116, because homozygous deletion of $p21^{WAF1}$ completely abrogates the G_1 checkpoint (16) and leads to a repair defect (17) after

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γ-irradiation of these cells. Recently, it has been shown that $p21^{WAF1}$ may also mediate G_2 arrest (18, 19). $p21^{WAF1}$ may also have a role in apoptosis. Cells lacking $p21^{WAF1}$ appear to undergo apoptosis normally (20, 21). In other systems, transfection of $p21^{WAF1}$ has been found to protect cells from apoptosis (22, 23). Inactivation of $p21^{WAF1}$ sensitizes colorectal cancer cells to apoptosis by p53 (23). These results suggest that $p21^{WAF1}$ 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 $p21^{WAF1}$ 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);[3] hence, MCF10A and MCF10ACA1a 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, $p21^{WAF1}$ and p53 had been knocked out by homologous recombination, respectively.

Cell Culture. All cells were cultured in 95% air, 5% CO_2 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 μg/ml streptomycin, 1 μg/ml insulin, 0.1 μg/ml choleratoxin, 0.5 μg/ml hydrocortisone (Sigma), 0.5 μg/ml Fungizone, and 0.02 μg/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, 100units/ml penicillin, and 100 μg/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 μg/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 μg/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 μg/ml hydrocortisone, 0.1 μg/ml cholera toxin, 5 μg/ml transferrin, 5 μg/ml insulin, and 2×10^{-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 μg/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 $p21^{WAF1}$ 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 1×10^5/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 μg/ml RNase + 200 μg/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 μg of proteins from whole cell lysates were mixed 1:1 with 2× 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× 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 μg) 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× SSC buffer and subsequently UV cross-linked in a Stratalinker (Stratagene, La Jolla, CA). The membrane was prehybridized and hybridized in 7 ml of hybridization buffer (0.25 M

[3] 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.

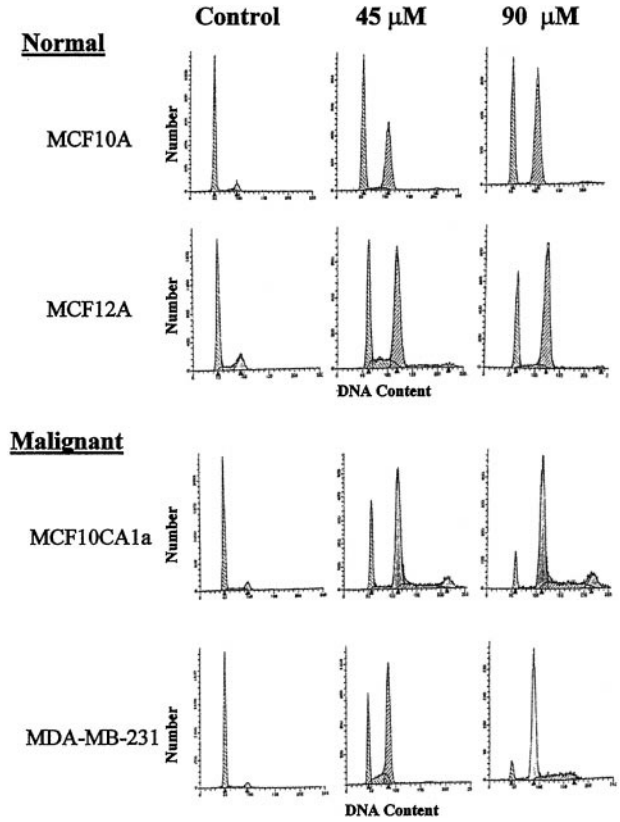

Fig. 1 Differential cell cycle effects of genistein between normal and malignant cells. Flow cytometric evaluation of cell cycle arrest in control and genistein-treated cells after 72 h. Numbers represent the percentage of cells in each phase of the cell cycle.

Na_2HPO_4 + 7% SDS) and ^{32}P-labeled $p21^{WAF1}$ 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 Na_2HP0_4 and 5% SDS at 65°C and again washed twice in 20 mM Na_2HP0_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 $p21^{WAF1}$ AS[4] 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 5×10^5/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 μl of DMEM/F-12, 3 μl of FuGENE 6, and 3 μg 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 recover for 48 h. After 48 h, hygromycin selection was started with the following concentrations: 100 and 700 μg/ml for MCF10A and MDA-MB-231 cells, respectively. Eighteen clones for each cell line were obtained and used for additional studies.

[4] The abbreviations used are: AS, antisense; PARP, poly(ADP-ribose) polymerase.

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

	G_0-G_1	S	G_2-M	Hyperdiploid
Nontumorigenic cell lines				
MCF10A				
Control	84	8	8	0
45 μM	50	5	45	0
90 μM	40	2	58	0
MCF12A				
Control	70	15	15	0
45 μM	31	16	53	0
90 μM	28	7	65	0
Tumorigenic cells				
MDA-MB-231				
Control	87	7	6	0
45 μM	24	16	58	2
90 μM	7	1	67	25
MCF10ACA1a				
Control	83	9	8	0
45 μM	20	2	53	25
90 μM	8	1	56	35
HN4				
Control	60	15	22	3
45 μM	14	22	52	12
90 μM	6	16	31	47
HTB9				
Control	75	19	5	0
45 μM	29	6	60	5
90 μM	14	4	70	12
H460				
Control	71	16	13	0
45 μM	26	20	49	5
90 μM	15	34	35	16

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 β-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 β-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_2-M cell cycle arrest in many different cancer cell lines. Treatment with genistein resulted in G_2-M cell cycle arrest in all cells tested. However, this effect

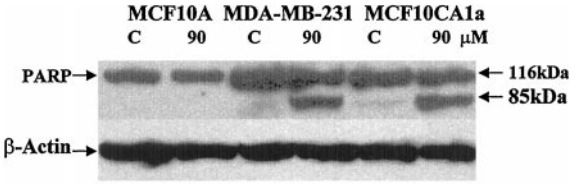

Fig. 2 Genistein induces differential apoptotic response between normal and malignant cells. Western blot analysis of PARP cleavage of control and genistein-treated cells after 72 h.

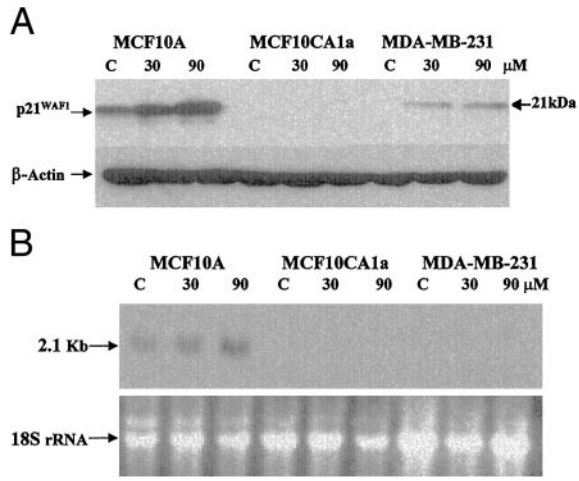

Fig. 3 *A,* differential induction of $p21^{WAF1}$ protein by genistein in normal and malignant cells. Western blot analysis of control and genistein-treated cells after 72 h. *B,* differential induction of $p21^{WAF1}$ mRNA by genistein in normal and malignant cells. Northern blot analysis of total RNA from 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_2-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_2-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_2-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_r 116,000 molecular weight protein, during apoptosis results in a M_r 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 $p21^{WAF1}$ Expression. $p21^{WAF1}$ has been shown to be modulated by genistein. $p21^{WAF1}$ has also been shown to play an important role in G_2-M cell cycle arrest and apoptosis. To determine whether $p21^{WAF1}$ may be differentially modulated in normal and malignant cells after genistein treatment, the levels of $p21^{WAF1}$ in control and genistein-treated MCF10A, MCF10CA1a, and MDA-MB-231 cells were examined. Three days after genistein treatment, $p21^{WAF1}$ protein levels increased 4–5-fold in genistein-treated MCF10A cells, compared with control cells (Fig. 3*A*). There was only a slight increase in $p21^{WAF1}$ levels in genistein-treated MDA-MB-231 cells, whereas no $p21^{WAF1}$ was detected in MCF10CA1a cells (Fig. 3*A*). These results suggest that genistein treatment of normal MCF10A cells results in a more pronounced induction of $p21^{WAF1}$ levels compared with malignant MDA-MB-231 and MCF10CA1a cells. We have shown that genistein modulates $p21^{WAF1}$ to a greater extent in MCF10A cells compared with MDA-MB-231 and MCF10CA1a cells. Modulation of $p21^{WAF1}$ by genistein could be attributable to changes in transcriptional activity of the $p21^{WAF1}$ promoter. Thus, the $p21^{WAF1}$ promoter might be differentially modulated by genistein in MCF10A, MCF10CA1a, and MDA-MB-231 cells, leading to differential modulation of $p21^{WAF1}$ protein in these cells.

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
MCF10ACA1a	
p21(+) untreated	79 ± 4.5
p21(+) treated	110 ± 7.5
MDA-MB-231	
p21(+) untreated	45 ± 7
p21(+) treated	97 ± 5

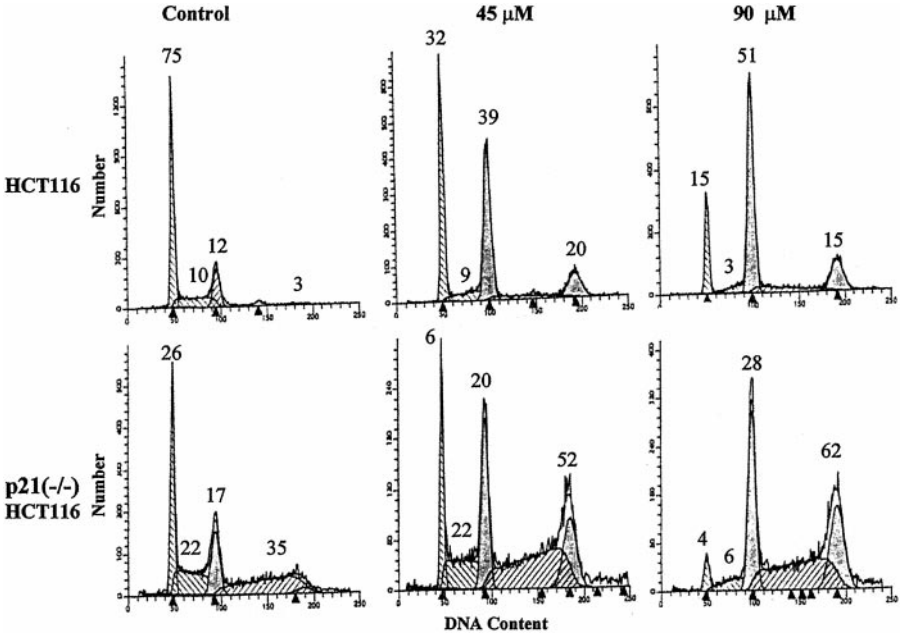

Fig. 4 Genistein induces a differential cell cycle effect in p21^{WAF1} −/− and parental HCT116 cells. Flow cytometric evaluation of cell cycle arrest in control and genistein-treated cells after 72 h is shown, as is cell cycle distribution of HCT116 and p21−/− cells after genistein treatment. Numbers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

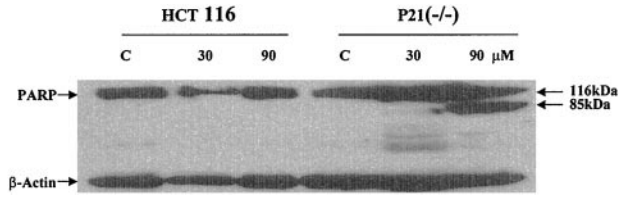

Fig. 5 Genistein induces differential apoptotic response in p21^{WAF1} −/− and parental HCT116 cells. Western blot analysis of PARP cleavage in control and genistein-treated cells after 72 h is shown.

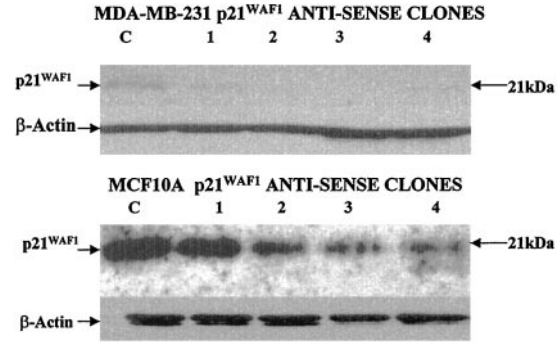

Fig. 6 Western blot analysis of p21^{WAF1} protein from cell lysates of control, MDA-MB-231 p21^{WAF1} AS clones, and MCF10A p21^{WAF1} AS clones.

Genistein Effects on p21^{WAF1} Promoter Activity. To determine the differential activation of the p21^{WAF1} promoter, we first examined p21^{WAF1} 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^{WAF1} mRNA in MCF10A cells, whereas there was a slight increase in p21^{WAF1} observed in MDA-MB-231 cells. We were unable to detect p21^{WAF1} RNA in either control or genistein-treated MCF10CA1a cells (Fig. 3*B*). 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^{WAF1} promoter. p53 is known to activate the p21^{WAF1} promoter. To determine whether activation of the p21^{WAF1} promoter by genistein is p53 dependent, we transfected the p21^{WAF1} 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^{WAF1} 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^{WAF1} promoter activity in MCF10A cells. Also, the basal promoter activity of wild-type p21^{WAF1} promoter was much greater compared with the p21^{WAF1} promoter without the p53 binding site. Only a modest activation of p21^{WAF1} promoter activity was observed in MCF10CA1a and MDA-MB-231 cells (Table 2). In summary, differential G_2-M cell cycle arrest and apoptosis between normal and malignant cells appear to be mediated by a differential effect of genistein on p21^{WAF1}. It appears that this process may be p53 dependent, which is the subject of our future investigation.

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

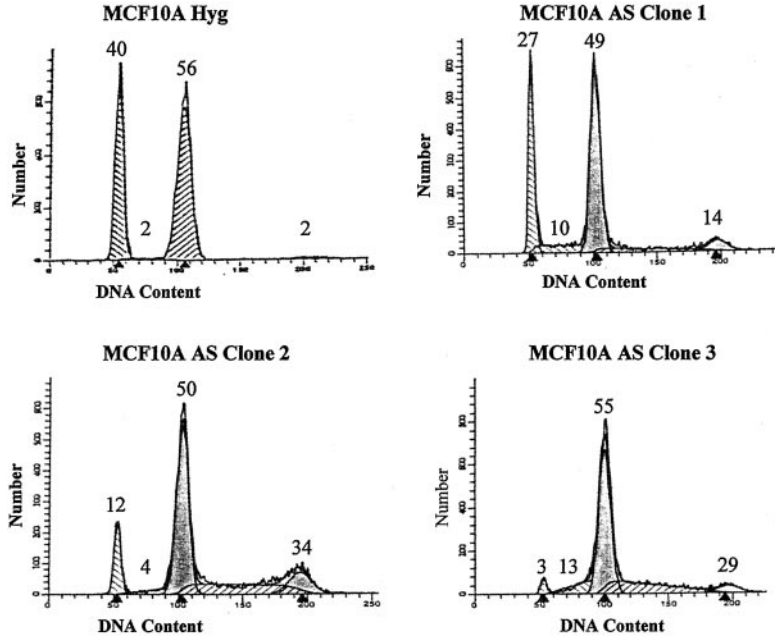

Fig. 7 Increased cell cycle arrest of MCF10A $p21^{WAF1}$ AS clones compared with control cells. Flow cytometric analysis of cell cycle distribution of MCF10A Hyg and $p21^{WAF1}$ AS clones after 90 μM genistein treatment for 72 h is shown. Numbers represent the percentage of cells in each phase of the cell cycle.

we performed flow cytometric analysis on genistein-treated $p21^{WAF1}$ −/− and $p21^{WAF1}$ +/+ HCT116 cells. We found that in both cell lines, there was an increase in G_2-M cell cycle arrest (Fig. 4). However, there were important differences observed. After 90 μM genistein treatment, $p21^{WAF1}$ −/− cells showed 4-fold fewer cells in G_0-G_1 compared with parental HCT116 cells (Fig. 4). Furthermore, genistein-treated $p21^{WAF1}$ −/− cells showed a 4-fold increase in the hyperdiploid population compared with HCT116 cells (Fig. 4). After 90 μM genistein treatment, there were ~25% more cells in the G_2-M and hyperdiploid phases of the cell cycle in $p21^{WAF1}$ −/− cells compared with parental HCT116 cells. These results suggest that $p21^{WAF1}$ is important for genistein mediated G_2-M arrest and that the lack of $p21^{WAF1}$ sensitizes cells to genistein-mediated cell cycle arrest.

Down-Regulation of $p21^{WAF1}$ Contributes to Genistein-mediated Apoptosis and Cell Cycle Arrest. To determine the role of $p21^{WAF1}$ 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 $p21^{WAF1}$ −/− cells compared with parental cells (Fig. 5). Visual observation did show some cell death in $p21^{WAF1}$ +/+ cells. Therefore, lack of $p21^{WAF1}$ appears to sensitize these cells to genistein-mediated apoptosis.

To further confirm the role of $p21^{WAF1}$ in sensitizing cells to genistein-mediated effects, we down-regulated $p21^{WAF1}$ by transfecting AS $p21^{WAF1}$ cDNA. Previously, we showed that $p21^{WAF1}$ is up-regulated in MCF10A and MDA-MB-231 cells after genistein treatment. Hence, we transfected these cells with the $p21^{WAF1}$ AS cDNA expression vector under the control of the cytomegalovirus promoter and a hygromycin-resistant control vector. After hygromycin selection, we obtained 18 $p21^{WAF1}$ AS clones of MCF10A and MDA-MB-231 cells. $p21^{WAF1}$ expression levels were determined by Western blot analysis (Fig. 6) in these clones. In MCF10A AS cells, $p21^{WAF1}$ expression was reduced 50−67% compared with parental cells. In MDA-MB-231 AS cells, $p21^{WAF1}$ expression was reduced 50−80% compared with parental cells. To determine whether $p21^{WAF1}$ 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 μM genistein caused 58% of the cells to arrest in the G_2-M phase of the cell cycle. Flow cytometric analysis showed that 84% of MCF10A $p21^{WAF1}$ AS clone 3 in the G_2-M and hyperdiploid phases of the cell cycle, after 90 μM genistein treatment (Fig. 7). Cells with greater $p21^{WAF1}$ down-regulation had increased proportion of cells arrested at G_2-M and a larger hyperdiploid population (Fig. 7). Similar results were obtained from MDA-MB-231 $p21^{WAF1}$ AS clones. Genistein treatment (45 μM) of MDA-MB-231 cells caused 60% of the cells to arrest in the G_2-M and hyperdiploid phases compared with 80% of MDA-MB-231 $p21^{WAF1}$ AS clone 2 after 45 μM genistein (Fig. 8). MDA-MB-231 AS clones with greater $p21^{WAF1}$ down-regulation showed greater growth arrest and hyperdiploid population (Fig. 8).

Because $p21^{WAF1}$ −/− colon carcinoma cells were more sensitive to apoptotic cell death compared with $p21^{WAF1}$ +/+ HCT116 cells after genistein treatment, we investigated whether a decrease in $p21^{WAF1}$ 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 $p21^{WAF1}$ AS clones with 90 μM genistein and examined PARP cleavage. Three days of genistein treatment did not induce apoptosis in MCF10A $p21^{WAF1}$ AS clones (Fig. 9). However, apoptosis-specific PARP cleavage was greatly enhanced in MDA-MB-231 $p21^{WAF1}$ AS clones compared with parental cells (Fig. 9). These results suggest that a partial down-regulation of $p21^{WAF1}$ in normal breast epithelial

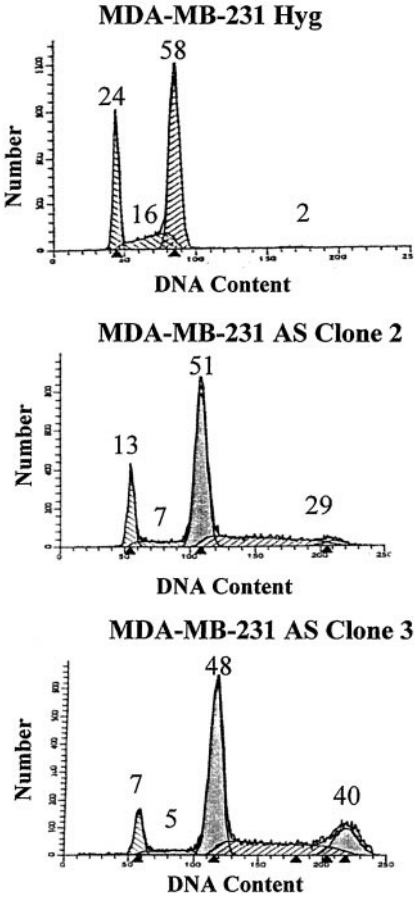

Fig. 8 Increased cell cycle arrest of MDA-MB-231 $p21^{WAF1}$ AS clones compared with control cells. Flow cytometric analysis of cell cycle distribution of MDA-MB-231 Hyg and $p21^{WAF1}$ AS clones after 45 μM genistein treatment for 72 h. Numbers represent the percentage of cells in each phase of the cell cycle.

cells (MCF10A) was not sufficient to trigger apoptosis. However, cancer cells could be sensitized to enhanced killing by down-regulation of $p21^{WAF1}$.

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.[5] 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 important for increased susceptibility of cancer cells to chemopreventive agents compared with normal cells.

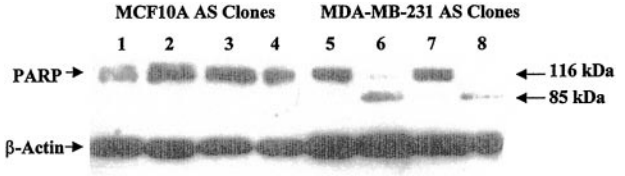

Fig. 9 Differential apoptotic response of MCF10A $p21^{WAF1}$ AS clones 2 and 3 and MDA-MB-231 $p21^{WAF1}$ AS clones 2 and 3 to genistein treatment. Western blot analysis of PARP cleavage in untreated (*Lanes 1, 3, 5,* and *7*) and genistein-treated (*Lanes 2, 4, 6,* and *8*) MCF10A and $p21^{WAF1}$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

In the present study, we showed that $p21^{WAF1}$ 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_2-M cell cycle arrest, hyperdiploid progression, and induction of apoptosis by genistein. Molecular profiling of these effects showed a greater induction of $p21^{WAF1}$ in normal cells compared with malignant cells. Therefore, we investigated whether $p21^{WAF1}$ plays any role in eliciting differential effects of genistein in normal and malignant cells.

We used $p21^{WAF1}$ −/− and $p21^{WAF1}$ +/+ HCT116 cells to further investigate the role of $p21^{WAF1}$ in genistein-mediated effects. Our studies revealed that $p21^{WAF1}$ −/− cells were more sensitive to cell cycle arrest and induction of apoptosis compared with $p21^{WAF1}$ +/+ cells. We further confirmed the role of $p21^{WAF1}$ in sensitizing cells to genistein by down-regulating $p21^{WAF1}$ 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 $p21^{WAF1}$ in cancer cells makes them more sensitive to genistein-mediated effects, compared with normal cells. Our $p21^{WAF1}$ 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 $p21^{WAF1}$ levels after genistein treatment.

Many studies have shown up-regulation of $p21^{WAF1}$ and subsequent apoptosis in various cancer cell lines by genistein (9, 30). Up-regulation of $p21^{WAF1}$ 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 $p21^{WAF1}$. Therefore, we believe that up-regulation of $p21^{WAF1}$ prevents apoptosis and induces cell cycle arrest. Certainly, it is possible that a combination of genetic make-up and up-regulation of $p21^{WAF1}$ 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 $p21^{WAF1}$ 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

[5] 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 $p21^{WAF1}$ 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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