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13. ABSTRACT ***(Maximum 200 Words)***

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 recently obtained experimental evidence supporting the differential effect of genistein in our system. We found that genistein causes a greater degree of G2/M arrest and induces apoptosis in malignant cell lines compared to 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, while its level was only slightly induced in malignant MDA-231 cells and not detectable in malignant MCF10CA1a cells. We also found that there was a greater degree of cell cycle arrest and apoptosis in $p21^{WAF1}$ -/- cells compared to $p21^{WAF1}$ +/+ 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. Moreover, we found that both malignant and normal $p21^{WAF1}$ anti-sense (AS) expressing clones became more sensitive to G2/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 epithelial AS clones. These results suggest that $p21^{WAF1}$ plays an important role in the differential effects of genistein in breast epithelial cells.

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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 of which tasks 1 through 4 has been successfully completed as acknowledged by the Department of Defense. In the last annual progress report we indicated that the following objectives to be investigated during the next reporting period. The objectives were:

- 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

Since the submission of our previous annual progress report, we conducted experiments to complete our stated objectives and the results of our studies and scientific activities during the last year are summarized in the following paragraphs. 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 have partly succeeded in accomplishing our overall goal, but to fully accomplish our goal, we obtained one year no cost extension approval from the Department of Defense. Therefore, this progress report is not the final report and thus, should be considered as annual 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.

BODY:

The following section describes the progress to-date of 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.

1. We have presented our overall data at the meeting organized by the Department of Defense (DOD) "Era of Hope" in Atlanta and the scientific abstract of which is presented below.

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, a protein tyrosine kinase inhibitor and a prominent isoflavonoid found in soy products appears to be the active agent responsible for lowering the rate of breast cancer in Asian women. However, the molecular mechanism(s) by which genistein elicits its effect on breast epithelial cells has not been fully elucidated. In this proposal, we investigated the effects of genistein on cell growth and apoptosis-related gene expression in breast epithelial cells. We found an up-regulation of Bax and $p21^{WAF1}$

expressions and down-regulation of Bcl-2, c-erbB-2 and reduced secretion of matrix metalloproteinases (MMPs) in genistein-treated breast cancer cells. Furthermore, DNA ladder formation, CPP32 activation, and PARP cleavage were also observed after treatment with genistein, which is consistent with the induction of apoptotic cell death observed by flow cytometric analysis of 7-amino actinomycin D stained cells. However, apoptosis was not observed in non-tumorigenic breast epithelial cells (MCF-10A) at concentrations of genistein that showed significant apoptotic cell death in cancer cell lines. From these results, we conclude that genistein inhibits the growth of MDA-MB-231 and MDA-MB-435 breast cancer cells, regulates the expression of cell cycle and apoptosis-related genes, and induces apoptosis through a p53-independent pathway. These molecular alterations may be the molecular mechanism(s) by which genistein induces cell growth inhibition and apoptosis in breast cancer cells, but not in non-tumorigenic breast epithelial cells. These results suggest that genistein may be a potentially effective preventive and/or therapeutic agent against breast cancer.

2. As indicated above that our objective was to further investigate the precise molecular mechanism(s) for the role of $p21^{WAF1}$ 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 has been recently submitted to "Oncogene" for its full publication (please see the attached manuscript). The summary of our results is also presented below as an abstract.

Abstract:

Genistein, a soy metabolite, is a potential chemopreventive agent against various types of cancer, however, the effect of genistein in normal epithelial cells has not been reported. In this study, we investigated whether genistein elicits differential effects on normal breast cell lines (MCF10A and MCF12A) and malignant (MCF10CA1a, MDA-231 and HCT116) cell lines. We found that genistein causes a greater degree of G2/M arrest and induces apoptosis in malignant cell lines compared to 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, while its level was only slightly induced in malignant MDA-231 cells and not detectable in malignant MCF10CA1a cells. Therefore, we investigated the role of $p21^{WAF1}$ in the differential effects of genistein among these cells. First we examined the effects of genistein on $p21^{WAF1}$ -/- and $p21^{WAF1}$ +/+ HCT116 cells. We found that there was a greater degree of cell cycle arrest and apoptosis in $p21^{WAF1}$ -/- cells compared to $p21^{WAF1}$ +/+ 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 that reduced $p21^{WAF1}$ levels enhance genistein mediated cell cycle arrest and apoptosis, we down-regulated $p21^{WAF1}$ by anti-sense $p21^{WAF1}$ cDNA transfection. We found that both malignant and normal $p21^{WAF1}$ anti-sense (AS) expressing clones became more sensitive to G2/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 epithelial AS clones. These results suggest that $p21^{WAF1}$ plays an important role in the differential effects of genistein in breast epithelial cells.

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 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 TO DATE:

- 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 $p21^{WAF1}$ in genistein-induced effects on breast epithelial cells

REPORTABLE OUTCOMES FOR THIS PERIOD:

- Presented data at the "Era of Hope" meeting
- Presented data at the annual meeting of AACR
- A manuscript has been submitted to "Oncogene"

CONCLUSIONS:

Collectively, we have accomplished Tasks 1 through 4 completely as indicated under the statement of work, and which the DOD has acknowledged. In addition, the results reported in the manuscript as indicated above provide 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 $p21^{WAF1}$ in mediating the biological effects of genistein on breast epithelial cells. Even though we have provided ample molecular evidence for the role of genistein in inhibiting the growth and inducing apoptotic processes in breast cancer cells, the question remains, "so what"? This question is partly answered as follows. 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. However, our *in vitro* results should be confirmed by *in vivo* animal or human studies in order to firmly establish the scientific rationale for the use of genistein in breast cancer prevention and/or treatment. In our future studies, we will fully complete our stated objectives.

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2. Upadhyay S, Neburi M, Sarkar FH. Role of p21WAF1 in differential response to genistein in MCF10A derived cells. Proceedings of the 91st annual meeting of the AACR. Volume 41, Page 845 (Abs # 5365), 2000.
3. Upadhyay S, Neburi M, Chinni S, Alhasan S, Miller F, Sarkar FH. Differential effects of genistein on normal and malignant cells is mediated by $p21^{WAF1}$. Oncogene (submitted, 2000).

APPENDICES:

1. Copy of the abstract presented at the "Era of Hope" meeting
2. Copy of the abstract presented at the 91st annual meeting of the AACR
3. Copy of the manuscript submitted to "Oncogene"

INDUCTION OF APOPTOSIS IN BREAST CANCER CELLS BY GENISTEIN

Fazlul H. Sarkar and Sunil Upadhyay

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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, a protein tyrosine kinase inhibitor and a prominent isoflavonoid found in soy products appears to be the active agent responsible for lowering the rate of breast cancer in Asian women. However, the molecular mechanism(s) by which genistein elicits its effect on breast epithelial cells has not been fully elucidated. In this proposal, we investigated the effects of genistein on cell growth and apoptosis-related gene expression in breast epithelial cells. We found an up-regulation of Bax and $p21^{WAF1}$ expressions and down-regulation of Bcl-2, c-erbB-2 and reduced secretion of matrix metalloproteinases (MMPs) in genistein-treated breast cancer cells. Furthermore, DNA ladder formation, CPP32 activation, and PARP cleavage were also observed after treatment with genistein, which is consistent with the induction of apoptotic cell death observed by flow cytometric analysis of 7-amino actinomycin D stained cells. However, apoptosis was not observed in non-tumorigenic breast epithelial cells (MCF-10A) at concentrations of genistein that showed significant apoptotic cell death in cancer cell lines. From these results, we conclude that genistein inhibits the growth of MDA-MB-231 and MDA-MB-435 breast cancer cells, regulates the expression of cell cycle and apoptosis-related genes, and induces apoptosis through a p53-independent pathway. These molecular alterations may be the molecular mechanism(s) by which genistein induces cell growth inhibition and apoptosis in breast cancer cells, but not in non-tumorigenic breast epithelial cells. These results suggest that genistein may be a potentially effective preventive and/or therapeutic agent against breast cancer.

This work was fully funded by a DOD grant awarded to FHS (DAMD17-97-1-7328)

no effect on dihydrotestosterone levels. Using reverse transcription-polymerase chain reaction (RT-PCR), genistein was found to cause a slight, but non-significant decrease in androgen receptor in the testis and DLP. Western blot analysis demonstrated no significant effect of genistein on AR in either tissue. RT-PCR and western blot analysis indicated a decrease in estrogen receptors-α and -β in both the DLP and testis. Aromatase mRNA was significantly reduced by both concentrations of genistein in the testis, but was undetectable in the DLP. There were no adverse effects on reproductive tract weights or body weight gain for any of the treatments used. The hormonal and signalling alterations following exposure to genistein in the diet may explain the lower incidence of clinically-manifested prostate cancer in Asian countries on a soy diet compared to other countries that lack substantial phytoestrogen intake.

#5364 CHEMOPREVENTIVE EFFECTS OF GENISTEIN ON METHYLNITROSOUREA (MNU)-INDUCED MAMMARY TUMORS. Ronald A Lubet, V E Steele, S Barnes, D L Hill, G J Kelloff, M M Juliana, and C J Grubbs, *National Cancer Inst, Bethesda, MD, Univ of Alabama at Birmingham, Birmingham, AL, and Univ of Florida, Gainesville, FL*

Epidemiologic evidence implies that diets high in soy isoflavones (including genistein) inhibit a variety of cancers; particularly those of the breast. In our first study, female Sprague-Dawley rats received either 2000 or 200 mg genistein/kg of diet beginning at 43 days of age. One week later, the rats were given MNU (50 mg/kg BW). At 100 days after the carcinogen, rats receiving the high and low doses of genistein had 61% and 44% fewer mammary tumors than the "carcinogen treated only" rats, respectively. The efficacy of genistein was also examined in a mammary cancer model in which rats were administered MNU (50 mg/kg BW) at 100 days of age. This model was used since it was hypothesized that genistein might alter mammary gland development and, thereby, not require continual exposure. When genistein (300 mg/kg diet) was started at 23 days of age and stopped one week prior to MNU, a slight enhancement of tumor growth was observed. Groups of rats in which genistein was administered both prior to and after MNU or only after MNU, however, had reduced mammary tumors. These studies suggest that genistein is effective in preventing mammary tumors induced by MNU, especially of tumors induced in younger rats. Supported by NCI N01-CN-85174-MAO.

#5365 ROLE OF $P21^{WAF1}$ IN DIFFERENTIAL RESPONSE TO GENISTEIN IN MCF10A DERIVED CELLS. Sunil Upadhyay, Madhavi Neburi, and Fazlul H Sarkar, *Karmanos Cancer Institute, Detroit, MI, and Karmanos Cancer Institute / Wayne State Univ, Detroit, MI*

Many studies have shown that genistein induces cell cycle arrest and apoptosis in different cell lines. We have previously shown that genistein induces growth arrest but not apoptosis in MCF10A untransformed breast epithelial cells. In this study, we show that CA1a cells, which are derived from MCF10A and are tumorigenic in nude mice, are more sensitive to genistein compared to MCF10A cells. Cell proliferation and flow-cytometric analysis were done to examine growth inhibitory effects. Dose-dependent growth inhibition was more pronounced in CA1a cells compared to MCF10A cells. We also observed an induction of apoptosis as measured by DNA ladder and PARP cleavage in genistein treated CA1a cells, but not in MCF10A cells. The cell growth inhibition and apoptosis have previously been shown to be mediated by $p21^{WAF1}$. We examined the expression of $p21^{WAF1}$ by Western blot analysis and found that $p21^{WAF1}$ was undetectable in CA1a cells, but its level increased several fold in genistein treated MCF10A cells, suggesting that $p21^{WAF1}$ may play an important role in the differential effects of genistein. To further examine the role of $p21^{WAF1}$ in genistein mediated effects, we used a $p21^{WAF1}$ knockout (k/o) colon cancer cell line (HCT116). By using flow-cytometric analysis, we found that $p21^{WAF1}$ k/o cells were more sensitive to genistein, compared to parental HCT116 cells. From these results, we conclude that the lack of $p21^{WAF1}$ may further sensitize cells to growth inhibitory and apoptotic effects of genistein.

#5366 EFFECTS OF ISOFLAVONES ON SIGNALING PATHWAYS IN RAT AND -HUMAN GLIOMA CELL LINES. Launa M J Lynch, S Khoshyomn, P L Penar, T R Tritton, and A Bhushan, *Haverford Coll, Haverford, PA, Idaho State Univ, Pocatello, ID, and Univ of Vermont, Burlington, VT*

Poor prognosis of patients with glioblastoma multiforme is due to the invasion and infiltration of the tumor cells into normal brain tissue. We have previously shown that genistein, at 5μM, can block invasion of glioblastoma multiforme into fetal rat brain aggregates (FBRA). Our studies also have shown that PLCγ-1 may be involved in the invasion of glioblastoma multiforme into FBRA. These studies were initiated to investigate the possibility that genistein and other soy bean-derived isoflavones exert their anti-tumor effects via actions on signal transduction mechanisms. In C6 rat glioma cells and U87 human glioma cells treated with 1, 5, and 50 μM concentrations of genistein for 72 hours, we determined the effect on PLCγ-1 and Erk-1. Erk-1 levels in cells treated with 50μM genistein is markedly decreased. Erk-1, JNK-1, and p38 levels was decreased in the U87 cells treated with 5 μM of diadzein. We hypothesize that glioma invasion may involve the MAP kinase pathways.

#5367 RESVERATROL, A NATURAL POLYPHENOLIC COMPONENT OF GRAPES AND RED WINES, INHIBITS INTESTINAL CARCINOGENESIS. Y Schneider, B Duranton, F Vincent, L Badolo, F Gosse, R Schleiffer, N Seiler, and F Raul, *Ircad, Strasbourg, France, and Univ Louis Pasteur, Strasbourg, France*

Resveratrol has chemopreventive properties but its effects on intestinal carcinogenesis have not been studied. In presence of 25μM resveratrol a 70% growth inhibition is observed in a human colonic cancer cell line (CaCo-2) associated with a cell cycle arrest at S/G2. When administered to *Min* mice (genetically predisposed to develop intestinal tumors), resveratrol (0.01% in drinking fluid for 7 wks) reduced by 70% the number of small intestinal tumors and prevented the formation of colonic tumors. The measure of the differential gene expression in the intestinal mucosa of control and resveratrol treated *Min* mice, showed that resveratrol caused the down-regulation of genes involved in cell cycle progression or cell proliferation (cyclins D1 and D2, Y-box binding protein). In addition, resveratrol up-regulated genes involved in the inhibition of tumorigenesis (protein TSG101, desmocollin 2) and in the recruitment and activation of immune cells (monocyte chemotactic protein 3, cytotoxic T lymphocyte Ag-4). Our results highlight the multiplicity of the molecular targets of resveratrol and the efficacy of its chemopreventive action.

#5368 SELECTIVE INHIBITION OF HUMAN CYTOCHROME P450 1A1 BY RESVERATROL AND ITS ANALOGUES. Young Jin Chun, M Y Kim, and F P Guengerich, *Chungang Univ, Seoul, Korea, and Vanderbilt Univ Sch of Medicine, Nashville, TN*

To investigate the mechanism of anticarcinogenic activities of resveratrol, a phytoalexin compound found in grape wine, the effects on cytochrome P450 were determined in human liver microsomes and *Escherichia coli* membranes coexpressing human P450 1A1 or P450 1A2 with human NADPH-P450 reductase (bicistronic expression system). Resveratrol slightly inhibited ethoxyresorufin *O*-deethylation (EROD) activity in human liver microsomes with an IC_{50} of 1.1 mM. Interestingly, resveratrol exhibited potent inhibition of human P450 1A1 in a dose-dependent manner with IC_{50} of 23 μM for EROD and IC_{50} of 11 μM for methoxyresorufin *O*-demethylation (MROD). However, the inhibition of human P450 1A2 by resveratrol was not so strong (IC_{50} 1.2 mM for EROD and 580 μM for MROD). Resveratrol showed over 50-fold selectivity for P450 1A1 over P450 1A2. In a human P450 1A1/reductase bicistronic expression system, resveratrol inhibited human P450 1A1 activity in a mixed-type inhibition (competitive-noncompetitive) with *Ki* values of 9 and 89 μM. The effects of various hydroxystilbenes that have a similar structure to resveratrol on P450 activities were determined. Among 8 compounds we tested, rhapontigenin (3,5,5′-trihydroxy-4′-methoxystilbene) showed the most selective inhibition of P450 1A1 over P450 1A2. These results suggest that resveratrol and its hydroxystilbene analogues are selective human P450 1A1 inhibitors, and may be considered for use as a strong cancer chemopreventive agent in humans.

#5369 RESVERATROL MODULATES THE EXPRESSION OF GENES INVOLVED IN THE METABOLISM OF PAH IN HUMAN BRONCHIAL EPITHELIAL CELLS. Steen Mollerup, S Ovrebo, and A Haugen, *National Institute of Occupational Health, Oslo, Norway*

The phytoalexin resveratrol (*trans*-3,4',5-trihydroxydistilbene), which is a diphenolic antioxidant found in plants and foods, has been suggested to have cancer chemopreventive and chemotherapeutic potential. A lower risk of tobacco-smoke induced lung cancer among consumers of wine compared to nondrinkers has been observed, which has been attributed to the high content of resveratrol particularly in red wine. We have studied the effect of resveratrol on the expression of the *CYP1A1*, *CYP1B1*, *mEH*, and *GSTP1* genes in an immortalized human bronchial epithelial cell line (BEP2D) by quantitative reverse transcriptase-PCR. The cells were treated either with benzo[a]pyrene (B[a]P) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), in the presence or absence of resveratrol. We found that resveratrol inhibited both constitutive and induced (B[a]P or TCDD) expression of the *CYP1A1* and *CYP1B1* genes dose-dependently. In contrast, the expression of *mEH* was increased in response to resveratrol. Apparently, resveratrol did not alter the expression of *GSTP1*. The altered gene expression in response to resveratrol was reflected in a reduced level of B[a]P metabolism. These data indicate that resveratrol may excert cancer chemopreventive activity through altering the expression of genes involved in the metabolism of PAH, which results in reduced formation of carcinogenic PAH metabolites. (Supported by the Norwegian Cancer Society)

#5370 MODULATION OF G2/M-SPECIFIC CELL CYCLE REGULATORY PROTEINS BY APIGENIN IS P21/WAF1 INDEPENDENT. M McVean, W C Weinberg, and J C Pelling, *NIH, Bethesda, MD, and Univ of Kansas Med Ctr, Kansas City, KS*

Apigenin, a nonmutagenic flavonoid, has been shown to inhibit UV-induced skin tumorigenesis in mice when topically applied, possibly by triggering G2/M arrest. We investigated the role of p21/waf1 in modulating cell cycle regulatory proteins during apigenin-induced G2/M arrest. We exposed p21/waf1 wild-type (WWT8) and p21/waf1 knock-out (WKO1 6) keratinocyte cell lines to apigenin for 24 hours and observed a G2/M arrest. p53 protein levels were increased after 4 hours of treatment with apigenin in a dose-dependent manner in both cell lines and p21/waf1 was induced afer 24 hours in WWT8 but not WKO16 keratinocytes. We then measured the effect of apigenin on cdc2 and cyclin B1 since these

DIFFERENTIAL EFFECTS OF GENISTEIN ON NORMAL AND MALIGNANT CELLS IS MEDIATED BY $P21^{WAF1}$

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Abstract

Genistein, a soy metabolite, is a potential chemopreventive agent against various types of cancer, however, the effect of genistein in normal epithelial cells has not been reported. In this study, we investigated whether genistein elicits differential effects on normal breast cell lines (MCF10A and MCF12A) and malignant (MCF10CA1a, MDA-231 and HCT116) cell lines. We found that genistein causes a greater degree of G2/M arrest and induces apoptosis in malignant cell lines compared to 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, while its level was only slightly induced in malignant MDA-231 cells and not detectable in malignant MCF10CA1a cells. Therefore, we investigated the role of $p21^{WAF1}$ in the differential effects of genistein among these cells. First we examined the effects of genistein on $p21^{WAF1}$ -/- and $p21^{WAF1}$ +/+ HCT116 cells. We found that there was a greater degree of cell cycle arrest and apoptosis in $p21^{WAF1}$ -/- cells compared to $p21^{WAF1}$ +/+ 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 that reduced $p21^{WAF1}$ levels enhance genistein mediated cell cycle arrest and apoptosis, we down-regulated $p21^{WAF1}$ by anti-sense $p21^{WAF1}$ cDNA transfection. We found that both malignant and normal $p21^{WAF1}$ anti-sense (AS) expressing clones became more sensitive to G2/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 epithelial AS clones. These results suggest that $p21^{WAF1}$ plays an important role in the differential effects of genistein in breast epithelial cells.

Introduction

Breast cancer is the leading cause of cancer related death in women in the United States. It represents 32% of all new cases and causes 18% of the cancer related deaths among women (Wood, 1994). Recent dietary and epidemiological studies have shown a benefit of a soy diet in the prevention of breast cancer (Adlercreutz, 1995; Knight & Eden, 1996). The soy metabolite, phytochemical genistein, has been implicated as the anti-cancer component of the soy diet. Possible mechanisms for the anti-proliferative property of genistein include: prevention of DNA mutation, reduction in cancer cell proliferation, inhibition of angiogenesis, and induction of differentiation (Knight & Eden, 1996). Epidemiological studies with Asian immigrants in the United States further suggest that susceptibility to breast cancer is partly due to environmental differences (especially diet) rather than genetic differences (Griffiths *et al.*, 1996). There is an association between decreased breast cancer risk and increased phytochemical consumption (Adlercreutz, 1995; Lee *et al.*, 1991). Women who consume soy milk regularly have reduced levels of endogenous ovarian and adrenal hormones which are recognized risk factors for breast cancer (Lu *et al.*, 1996). Urinary excretion of phytochemicals directly correlates with consumption (Adlercreutz *et al.*, 1987). As expected, low urinary excretion of phytochemicals is observed in women at high risk for breast cancer (Adlercreutz *et al.*, 1986; Adlercreutz *et al.*, 1982), with the lowest urinary excretion of phytochemicals being from women with breast cancer (Rose, 1990). 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 (Barnes *et al.*, 1990; Hawrylewicz *et al.*, 1991).

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 (Peterson & Barnes, 1991; Li, *et al.*, 1999). It is generally accepted that genistein can arrest the cells at G2/M (Pagliacci *et al.*, 1994), but a recent report has shown that genistein can also cause G0/G1 arrest in a mouse fibroblast cell line (Kuzumaki *et al.*, 1998). Collectively, these reports suggest that cell cycle arrest caused by genistein may be due to both G0/G1 and G2/M arrest, depending on the cell lines and experimental conditions. However, such studies have not used normal breast epithelial cells.

Anti-proliferative actions of chemo-preventive agents, including genistein, may be mediated by up-regulation of $p21^{WAF1}$ (Lallemand *et al.*, 1999; Lin *et al.*, 1999; Li, *et al.*, 1999). $P21^{WAF1}$ expression is usually controlled at the transcriptional level by both p53-dependent and p53–independent mechanisms. Transient expression of $p21^{WAF1}$ results in inhibition of growth and DNA synthesis (el-Deiry *et al.*, 1993; Harper *et al.*, 1993). Introduction

of $p21^{WAF1}$ expression constructs into normal (Harper *et al.*, 1993) and tumor cell lines (el-Deiry *et al.*, 1993) results in cell cycle arrest in G1 (Harper *et al.*, 1995). $P21^{WAF1}$ appears to be solely responsible for G1 arrest in human colon carcinoma cell line HCT116, since homozygous deletion of $p21^{WAF1}$ completely abrogates the G1 checkpoint (Waldman *et al.*, 1995) and leads to a repair defect (McDonald *et al.*, 1996) following γ-irradiation of these cells. Recently, it has been shown that $p21^{WAF1}$ may also mediate G2 arrest (Dulic *et al.*, 1998; Niculescu *et al.*, 1998). $P21^{WAF1}$ may also have a role in apoptosis. Cells lacking $p21^{WAF1}$ appear to undergo apoptosis normally (Brown *et al.*, 1997; Deng *et al.*, 1995). In other systems, transfection of $p21^{WAF1}$ has been found to protect cells from apoptosis (Gorospe *et al.*, 1997; Polyak *et al.*, 1996). Inactivation of $p21^{WAF1}$ sensitizes colorectal cancer cells to apoptosis by p53 (Gorospe & Holbrook, 1996; Polyak *et al.*, 1996). Furthermore, it has been shown that $p21^{WAF1}$ can form a complex with pro-caspase-3 and Caspase-3 contains a $p21^{WAF1}$ binding domain in N-terminus (Suzuki *et al.*, 1999a; Suzuki *et al.*, 1999b). This suggests that $p21^{WAF1}$ plays a role in activation of caspases, which are important mediators of apoptosis.

Genistein selectively inhibits proliferation of Ras-transformed NIH 3T3 cells, but not non-transformed NIH 3T3 cells. In addition, transformation frequencies can be reduced by genistein: fewer NIH 3T3 cells are transformed by mutant RAS in the presence of genistein (Okura *et al.*, 1988). Yet, there have been no studies documenting the differential effect of genistein in normal and malignant epithelial cells. The purpose of this study was to examine whether there is any differential effect of genistein in normal and malignant breast epithelial cells. Since $p21^{WAF1}$ is involved in both growth regulation and cell death, we examined the role of $p21^{WAF1}$ in differential effects of genistein among normal and malignant epithelial cells.

Material 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 lines that were derived without viral or chemical intervention from mortal diploid human breast epithelial cells (Soule *et al.*, 1990). The characteristics of this cell line and tissue culture conditions are well established (Tait *et al.*, 1990). MCF12A was derived from mortal diploid cells obtained from different patient than the MCF10A donor (Wolman *et al.*, 1994). MCF10A and MCF12A cells contain wild-type p53 gene. MCF10CA1a cells were derived from MCF10NeoT model system (Miller *et al.*, 1993; Santner *et al.*, 2000). HCT116, 80S14, and 379.2 are colon cancer cell lines that were obtained from Dr. Bert Vogelstein at Johns Hopkins University. 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) media (Gibco/BRL) supplemented with 5% horse serum (Gibco/BRL), 2mM L-glutamine, 100 units/ml penicillin, 100μg/ml streptomycin, 1μg/ml insulin, 0.1μg/ml choleratoxin, 0.5μg/ml hydrocortisone (sigma), 0.5μg/ml fungizone, and 0.02μg/ml EGF (Gibco/BRL).

MCF10CA1a cells were cultured in DMEM/F-12 (1:1) media supplemented with 5% horse serum, 2mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. MCF10A, MCF12A, and MCF10CA1a cells were obtained from Karmanos Cancer Institute, Detroit, Michigan.

MDA-231 cells were cultured in DMEM/F-12 (1:1) media supplemented with 10% fetal bovine serum (Gibco/BRL), 2mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

HCT116, 80S14, and 379.2 cells were cultured in McCoys 5A media supplemented with 10% fetal bovine serum (Gibco/BRL), 2mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Antibodies

Mouse anti-human $p21^{WAF1}$ antibody was purchased from Pharmingen (Lexington, KY). Mouse anti-poly (ADP-ribose) polymerase monoclonal antibody was purchased from BIOMOL INC (Plymouth Meeting, PA).

Flow Cytometry

Cells were seeded at a density of 1 x 10^5/well in six-well culture dishes. The cells were treated with various concentrations of genistein for three days and harvested by trypsinization. The cells were centrifuged at 2,000 rpm for five minutes, washed with phosphate-buffered saline (PBS), and then fixed with 70% ethanol for at least 4 hours. After fixation, cells were centrifuged at 2,000 rpm, washed with PBS, and centrifuged again. Cell pellets were suspended in 1ml of PBS with 0.1% Triton X-100 + 200 µg/ml RNAse + 200 µg/ml propidium iodide for at least 1 hour. 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, 125mM Tris-HCl, pH 6.8, 20% Glycerol). Protein concentrations were measured using a commercial protein assay reagents (Pierce, IL) to ensure equal loading. 20 µg of proteins from whole cell lysates were used for each lane. Samples were mixed 1:1 with 2Xsample buffer and then applied to 10-14% polyacrylamide gels. Samples were electrophoretically separated and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). Following transfer, the membranes were blocked by 5% fat-free milk in 20 ml 1xTTBS (0.2% Tween-20, 136 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl, pH 7.4) for 1 hour and then incubated with 5 ml of specific primary antibodies in milk with rotation for 2 hours at room temperature. The membranes were washed 3 times for 15 minutes each with 1xTTBS solution, then incubated with anti-mouse or anti-rabbit peroxidase-conjugated secondary antibodies (1:3000) (Bio-Rad, CA) for 2 hours with rotation at room temperature. Following the incubation, the membranes were washed 3 times for 15 minutes each with 1xTTBS solution and then incubated with 2ml of chemiluminescence reagent (Pierce, IL). The protein bands were visualized using X-ray films (Kodak, NY).

RNA Isolation

Total cellular RNA was isolated using Trizol reagent (Gibco/BRL). Cells were transferred into eppendorf tubes and then lysed with 1 ml of Trizol reagent for 5 minutes at room temperature. 0.2 ml of chloroform was added, and tubes were shaken vigorously for 15 sec, and incubated for 2-3 min at room temperature. After centrifugation for 15 min at 4°C, supernatant was transferred to new tubes, mixed with 0.5ml of isopropanol, incubated for 10 min at room temp, and centrifuged at 12,000xg for 10 min at 4°C. Pellets were washed with 75% ethanol, air dried, and dissolved in nuclease free water.

Northern Blot Analysis

RNA (10 µg) from MCF10A, MCF10CA1a, and MDA-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 20xSSC buffer and subsequently UV cross-linked in a Stratalinker (Stratagene, La Jolla, CA). The membrane was prehybridized for one hour in 7 ml of hybridization buffer (0.25M Na_2HP0_4 + 7% SDS). Northern blot analysis was carried out by hybridization with specific ^{32}P labeled $p21^{WAF1}$ probe overnight at 65°C in hybridization buffer at constant rotation. Following hybridization, the membrane was then washed twice (30 minutes each) in 20mM Na_2HP0_4 and 5% SDS at 65°C and again washed twice in 20mM 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.

Labeling $p21^{WAF1}$ cDNA with [^{32}P]ATP

The random priming reaction was carried out using the Prime-It II kit (Stratagene, CA) and $p21^{WAF1}$ cDNA. The reaction was incubated for 10 min. at 37°C and stopped by the addition of 0.5 M EDTA followed by the addition of STE buffer. The reaction was loaded on to a chromatography column (Stratagene) in order to remove

unincorporated radio-nucleotides. 2 µl of the probe was used to determine specific activity using the scintillation counter.

PARP Assay

Cells were washed twice with ice-cold PBS and harvested by scraping the cells with a rubber policeman in ice-cold PBS. Whole cell extraction buffer (WCE buffer, containing 50mM Tris-HCl, 137 mM NaCl, 2mM EDTA, 10mM EGTA, 1 % Triton X-100, 50mM beta-glycerophosphate, 5mM $MgCl_2$, 1mM Na_3VO_4, 1 mM DTT and double distilled water containing protease inhibitors leupeptin, pepstain, aprotinin and PMSF) was added to the cell pellet and collected in an eppendorf tube. Samples were allowed to sit on ice for 30 min. The tubes were centrifuged at 4°C for 5 min. After spinning, supernatant was transferred to a new tube, the protein concentration quantitated and the lysates was stored at -80°C. Protein samples were subjected to Western blot analysis using 10% SDS-PAGE and transferred to nitrocellulose membranes. Target was detected using ECL Western detection system, with mouse monoclonal anti-PARP as primary and HRP-conjugated goat anti-mouse as secondary antibodies.

Gene Transfection Studies

MCF10A and MDA-231 cells were transfected with $p21^{WAF1}$ anti-sense (AS) cDNA, a gift from Dr. Vogelstein at Johns Hopkins University. The cDNA is under the control of cytomegalovirus promoter was introduced into these cells using Fugene 6 (Boehringer Mannheim) reagent. Cells were plated in 100mm dishes at a density of $5x10^5$/ dish. The following day, each culture dish was washed with PBS solution and overlaid with serum-free DMEM/F-12 medium. In a small sterile tube, 100µl of DMEM/F-12, 3µl of Fugene 6, and 3 µg of DNA were mixed and incubated for 20 minutes. Then it was transferred into the dishes containing serum-free media and allowed to react with the cells overnight. On the next day, cells were replenished with complete media and allowed to recover for 48 hours. After 48 hours, hygromycin selection was started with the following concentrations: 100 µg/ml and 700 µg/ml for MCF10A and MDA-231 cells, respectively. Eighteen clones for each cell line were obtained and used for further studies.

Luciferase Assay

$5X10^5$ cells per well were plated 24 hours prior to transfection in 6-well plates. Next day, luciferase DNA constructs and β-galactosidase expression plasmid pCH100 were transfected per well for 24 hours in serum free media, using Fugene 6 (Boehringer Mannheim). On the next day, complete media was added and 24 hours later, cells were treated with genistein for 24 hours. Cells were lysed in reporter lysis buffer (Promega, WI) and lysates were assayed for luciferase activity using luminometer. Lysates were also assayed for β-galactosidase activity to normalize for transfection efficiency.

Results

In order to determine whether there are any differential effects of genistein between normal and malignant cells, we treated immortalized normal breast epithelial cell lines MCF10A and MCF12A, and malignant cell lines MCF10CA1a and MDA-231, with various concentrations of genistein and evaluated cell cycle arrest by flow-cytometric analysis. Genistein is known to cause G2/M cell cycle arrest in many different cancer cell lines. Treatment with genistein resulted in G2/M cell cycle arrest in all cells tested. However, this effect was much more pronounced in malignant cells compared to normal cells (figure 1). Interestingly, there was an appearance of a hyperdiploid population in malignant cells, after genistein treatment which was not observed in normal cells (figure 1). In normal cells, there were 58% (MCF10A) and 62% (MCF12A) cells in G2/M phase of the cell cycle after 90 µM of genistein treatment. Hyperdiploid population was not detected in normal cells. On the other hand, in malignant cells, there were 91% (MCF10CA1a) and 92% (MDA-231) in G2/M and hyperdiploid phase after 90 µM of genistein treatment (Table 1). We have previously reported a similar effect of genistein in a head and neck, and lung cancer cell lines, respectively (Alhasan, *et al.,* 2000; Lian, *et al.,* 1999). These malignant cell lines also exhibited hyperdiploid populations after genistein treatment.

Genistein is known to induce apoptosis in many different cancer cells lines (Pagliacci, *et al.,* 1994; Shao, *et al.,* 1998). A cascade of events whereby proteases such as caspase-3, are cleaved marks apoptotic processes. This activated caspase-3 then cleaves substrates such as poly-ADP-Ribose polymerase (PARP). Cleavage of PARP, a 116kDa molecular weight protein, during apoptosis results in 85kDa product, which can be visualized by Western

blot analysis. In order to determine whether there is any differential apoptotic response in normal and malignant cells, we examined PARP cleavage after genistein treatment. Three days after 90 μM of genistein treatment, an apoptotic cleavage fragment of PARP was readily detected in malignant MDA-231 and MCF10CA1a cells, but not in normal MCF10A (figure 2) and MCF12A cells (data not shown). These results suggest that malignant cells are more sensitive to genistein mediated cell cycle arrest and induction of apoptosis compared to normal cells.

$P21^{WAF1}$ has been shown to be modulated by genistein. $P21^{WAF1}$ has also been shown to play an important role in G2/M cell cycle arrest and apoptosis. In order to determine whether $p21^{WAF1}$ may be differentially modulated in normal and malignant cells after genistein treatment, we examined the levels of $p21^{WAF1}$ in control and genistein treated MCF10A, MCF10CA1a, and MDA-231 cells. Three days after genistein treatment, $p21^{WAF1}$ protein levels increased 4 to 5 fold in genistein treated MCF10A cells, compared to control cells (figure 3). There was only a slight increase in $p21^{WAF1}$ levels in genistein treated MDA-231 cells, while no $p21^{WAF1}$ was detected in MCF10CA1a cells (figure 3). These results suggest that genistein treatment of normal MCF10A cells results in a more pronounced induction of $p21^{WAF1}$ levels compared to malignant MDA-231 and MCF10CA1a cells.

We have shown that genistein modulates $p21^{WAF1}$ to a greater extent in MCF10A cells compared to MDA-231 and MCF10CA1a cells. Modulation of $p21^{WAF1}$ by genistein could be due 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-231 cells, leading to differential modulation of $p21^{WAF1}$ protein in these cells.

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-231 cells, using Northern blot analysis. Genistein treatment resulted in a 3 to 4 fold increase in $p21^{WAF1}$ mRNA in MCF10A cells, while there was a slight increase in $p21^{WAF1}$ observed in MDA-231 cells. We were unable to detect $p21^{WAF1}$ RNA in either control or genistein treated MCF10CA1a cells (figure 4). Since promoter activation can lead to changes in RNA levels, we performed the luciferase assay in control and genistein treated MCF10A, MCF10CA1a, and MDA-231 cells, using the $p21^{WAF1}$ promoter. Genistein treatment resulted in a 10- fold activation of $p21^{WAF1}$ promoter in MCF10A cells while only a modest activation of $p21^{WAF1}$ promoter was observed in MCF10CA1a and MDA-231 cells (Table 2). In summary, differential G2/M cell cycle arrest and apoptosis between normal and malignant cells appears to be mediated by a differential effect of genistein on $p21^{WAF1}$.

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, in which both copies of the $p21^{WAF1}$ was knocked out by homologous recombination, and the parental HCT116 cells. To determine whether a lack of $p21^{WAF1}$ sensitizes cells to cell cycle arrest, 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 G2/M cell cycle arrest (figure 5). However, there were important differences observed. After 90 μM of genistein treatment, $p21^{WAF1}$ -/- cells showed 4-fold fewer cells in G0/G1 compared to parental HCT116 cells (figure 5). Furthermore, genistein treated $p21^{WAF1}$-/- cells showed a 4-fold increase in the hyperdiploid population compared to HCT116 cells (Table 3). After 90 μM of genistein treatment, there were approximately 25% more cells in G2/M and hyper-diploid phase of cell cycle in $p21^{WAF1}$ -/- cells compared to parental HCT116 cells. These results suggest that $p21^{WAF1}$ is important for genistein mediated G2/M arrest. Furthermore, lack of $p21^{WAF1}$ sensitizes cells to genistein mediated cell cycle arrest.

In order to determine the role of $p21^{WAF1}$ in genistein mediated apoptosis, we investigated PARP cleavage. Three days after genistein treatment, the level of 85 kDa apoptotic cleavage fragment of PARP was much greater in $p21^{WAF1}$ -/- cells compared parental cells (figure 6). 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 anti-sense $p21^{WAF1}$ cDNA. Previously, we showed that $p21^{WAF1}$ is up-regulated in MCF10A and MDA-231 cells after genistein treatment. Hence, we transfected these cells with $p21^{WAF1}$ anti-sense (AS) cDNA expression vector under the control of cytomeglovirus promoter and a hygromycine resistant control vector. After hygromycin selection, we obtained 18 $p21^{WAF1}$ AS clones of MCF10A and MDA-231 cells. $P21^{WAF1}$ expression levels were determined by Western blot analysis (figure 7) in these clones. In MCF10A AS cells, $p21^{WAF1}$ expression was reduced 50 to 67% compared to parental cells. In MDA-231 AS cells, $p21^{WAF1}$ expression was reduced 50 to 80% compared to 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 previously shown that 90 μM genistein caused 58% of the cells to arrest in G2/M phase of the cell cycle. Flow cytometric analysis showed that

84% of MCF10A $p21^{WAF1}$ AS clone 3 in G2/M and hyperdiploid phase of the cell cycle, after 90 μM of genistein treatment (table 4). Cells with greater $p21^{WAF1}$ down-regulation had increased proportion of cells arrested and a larger hyperdiploid population (Table 4). Similar results were obtained from MDA-231 $p21^{WAF1}$ AS clones. 45 μM genistein treatment of MDA-231 cells caused 60% of the cells to arrest in G2/M and hyperdiploid phase compared to 80% of MDA-231 $p21^{WAF1}$ AS clone 2 after 45 μM genistein (table 5). MDA-231 AS clones with greater $p21^{WAF1}$ down-regulation showed greater growth arrest and hyperdiploid population (Table 5).

Since $p21^{WAF1}$ -/- colon carcinoma cells were more sensitive to apoptotic cell death compared to $p21^{WAF1}$ +/+ HCT116 cells after genistein treatment, we investigated whether a decrease in $p21^{WAF1}$ in the MCF10A normal breast and MDA-231 malignant breast cells could also enhance sensitivity to apoptotic cell death when exposed to genistein. We treated MCF10A and MDA-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 (Figure 10). However, apoptosis specific PARP cleavage was greatly enhanced in MDA-231 $p21^{WAF1}$ AS clones compared to parental cells (figure 10). These results suggest that a partial down-regulation of $p21^{WAF1}$ in MCF10A cells was not sufficient to trigger apoptosis. However, cancer cells could be sensitized to better killing by down-regulation of $p21^{WAF1}$.

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Discussion

The selective inhibition of Ha-Ras transformed NIH3T3 (Okura *et al.*, 1988) 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 (Kang *et al.*, 2000). 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 to normal cells.

In the present study, we show that $p21^{WAF1}$ plays an important role in eliciting differential effects of genistein between normal and malignant cells. We have shown that malignant cells are more sensitive to G2/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 to 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 to $p21^{WAF1}$ +/+ 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-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 to 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 which could be the basis for differential induction of $p21^{WAF1}$ level after genistein treatment.

Many studies have shown up-regulation of $p21^{WAF1}$ and subsequent apoptosis in various cancer cell lines by genistein (Pagliacci *et al.*, 1994; Shao *et al.*, 1998; Li, *et al.*, 1999; Alhasan *et al.*, 2000; Lian *et al.*, 1999). Up-regulation of $p21^{WAF1}$ observed in these cell lines are, perhaps, due to 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. Several studies have documented that apoptosis may occur due to $p21^{WAF1}$ over-expression (Chen *et al.*, 1996). There may be different forms of $p21^{WAF1}$, which are distributed differently in normal and malignant cells (Thomas *et al.*, 1998). Certain forms of these molecules may be functionally inactive and these may be predominant in cancer cells. Genistein mediated up-

regulation of $p21^{WAF1}$ may induce the inactive form of $p21^{WAF1}$, thus compromising the function of active $p21^{WAF1}$ molecules.

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 progression may occur in malignant cells that contribute to cell death is via modulation of apoptosis regulators such as Bcl-2, Bax, and caspases (Adams & Cory, 1998; Thornberry & Lazebnik, 1998). Hyperdiploid cells may be further sensitized to genistein effects due to lack of repair (McDonald *et al.*, 1996). Cell cycle regulators, such as Rb, CDC25C and 14-3-3σ (Hermeking *et al.*, 1997; Lopez-Girona *et al.*, 1999; Notterman *et al.*, 1998) also play important roles in cell cycle arrest and, therefore, may be differentially modulated in normal and malignant cells. Further studies with a combination of factors, such as Ha-Ras, $p21^{WAF1}$ down-regulation, and Rb mutation may provide a more comprehensive understanding of chemopreventive activity of genistein *in vivo*. In summary, our data suggests that genistein may have wide therapeutic applications because of its selective inhibition of malignant cells without any significant effect on normal cells.

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Figure Legends

Figure 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 hours.

Figure 2. Genistein has differential apoptotic response between normal and malignant cells. Western blot analysis of PARP cleavage of control and genistein treated cells after 72 hours.

Figure 3. Induction of $p21^{WAF1}$ in normal and malignant cells. Western blot analysis of control and genistein treated cells after 72 hours.

Figure 4. Differential induction of $p21^{WAF1}$ RNA by genistein in normal and malignant cells. Northern blot analysis of total RNA from control and genistein treated cells after 72 hours.

Figure 5. Genistein has 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 hours.

Figure 6. Genistein has differential apoptotic response in $p21^{WAF1}$ -/- and parental HCT116 cells. Western blot analysis of PARP cleavage in control and genistein treated cells after 72 hours.

Figure 7. Western blot analysis of $p21^{WAF1}$ protein from cell lysates of (a) control and MCF10A $p21^{WAF1}$ AS clones and (b) control and MDA-231 $p21^{WAF1}$ AS clones.

Figure 8. Increased cell cycle arrest of MCF10A $p21^{WAF1}$ AS clones compared to control cells. Flow cytometric analysis of control and 90 μM genistein treated cells after 72 hours.

Figure 9. Increased cell cycle arrest of MDA-231 $p21^{WAF1}$ AS clones compared to control cells. Flow cytometric analysis of control and 45 μM genistein treated cells after 72 hours.

Figure 10. Differential apoptotic response of MCF10A $p21^{WAF1}$ AS and MDA-231 $p21^{WAF1}$ AS clones to genistein treatment. Western blot analysis of PARP cleavage in control and genistein treated (a) MCF10A and $p21^{WAF1}$ AS clones and (b) MDA-231 and $p21^{WAF1}$ AS clones after 72 hours.

Table 1. Cell cycle distribution of various cell lines after genistein treatment. Numbers represent percentage of cells in each phase of cell cycle.

Non-tumorigenic Cell Lines

MCF10A	G0/G1	S	G2/M	HYPERDIP
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-231				
Control	87	7	6	0
45 μM	24	16	58	2
90 μM	7	1	67	25
CA1a				
Control	83	9	8	0
45 μM	20	2	53	25
90 μM	8	1	56	35

Table 2. Average luciferase activity (Mean+/- SD) in Normal and malignant cells.

MCF10A

p21(+) Untr	552 +/- 77
p21(+) Tr	5448 +/- 413

CA1a

p21(+) Untr	79 +/- 4.5
p21(+) Tr	110 +/- 7.5

MDA-231

p21(+) Untr	45 +/- 7
p21(+) Tr	97 +/- 5

Table 3. Cell cycle distribution of HCT116 and p21(-/-) cells after genistein treatment. Numbers represent percentage of cells in each phase of cells cycle.

	G0/G1	S	G2/M	HYPERDIP
HCT116				
Control	75	10	12	3
45 μM	32	9	39	20
90 μM	15	3	51	15
P21-/-				
Control	26	22	17	35
45 μM	6	22	20	52
90 μM	4	6	28	62

Table 4. Cell cycle distribution of MCF10A Hyg and p21 AS clones after genistein treatment. Numbers represent percentage of cells in each phase of cell cycle.

	G0/G1	S	G2/M	HYPERDIP
MCF10A Hyg 90 μM	40	2	58	0
MCF10A Cl 1 90 μM	27	10	49	14
MCF10A Cl 2 90 μM	12	4	50	34
MCF10A Cl 3 90 μM	3	13	55	29

Table 5. Cell cycle distribution of MDA-231 Hyg and p21 AS clones after genistein treatment. Numbers represent percentage of cells in each phase of cell cycle.

	G0/G1	S	G2/M	HYPERDIP
MDA-231 Hyg 45 μM	24	16	58	2
MDA-231 Cl 2 45 μM	13	7	51	29
MDA-231 Cl 3 45 μM	7	5	48	40

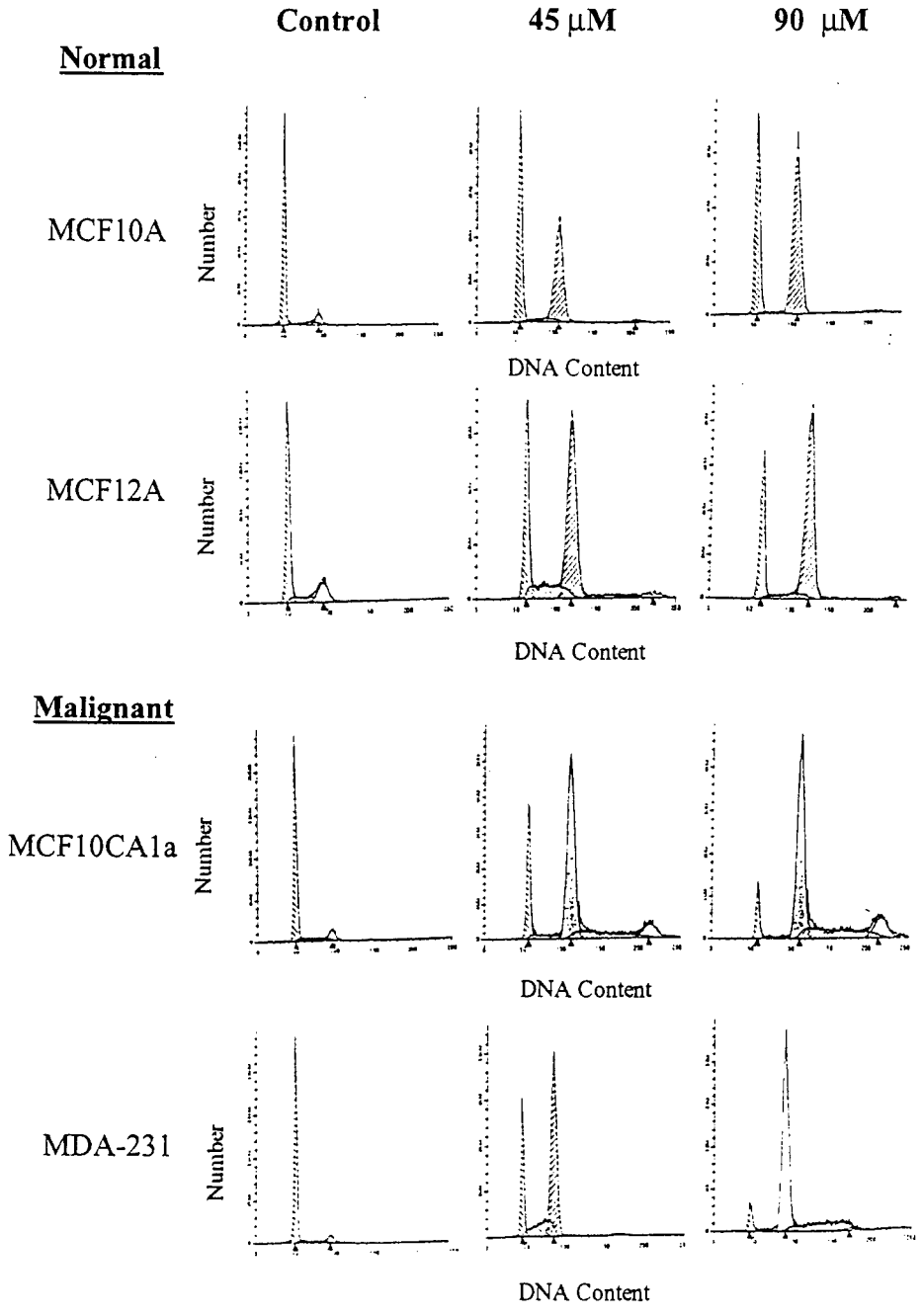

Control
45 μM
90 μM
Normal
MCF10A
Number
DNA Content
MCF12A
Number
DNA Content
Malignant
MCF10CA1a
Number
DNA Content
MDA-231
Number
DNA Content

FIGURE 1

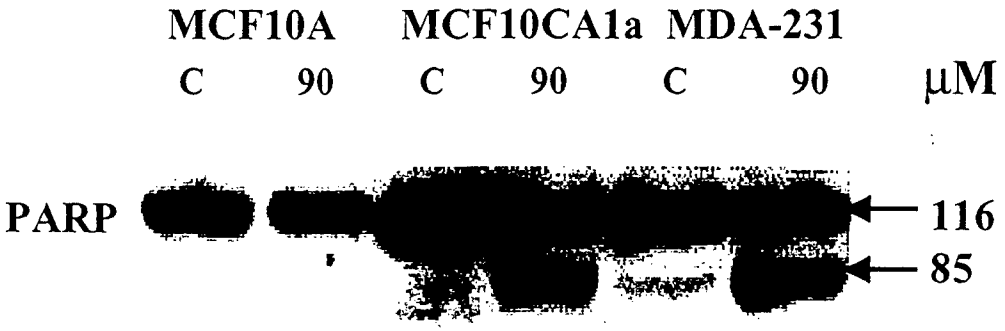


FIGURE 2

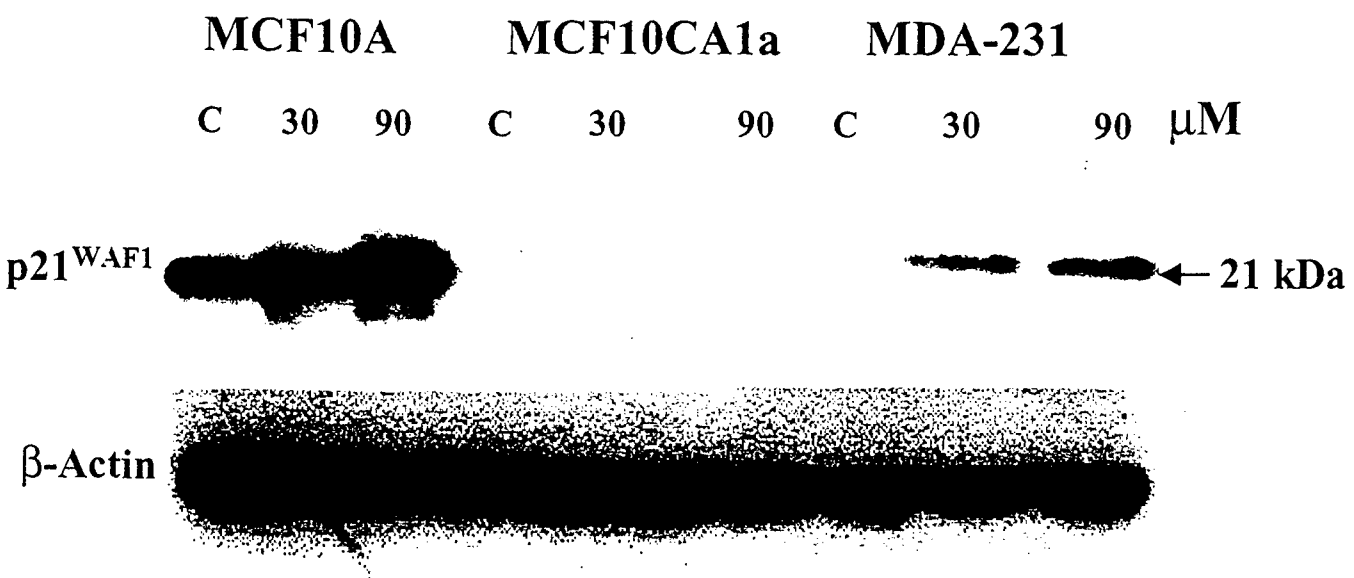

MCF10A
MCF10CA1a
MDA-231
C 30 90 C 30 90 C 30 90 μM
p21WAF1
21 kDa
β-Actin

FIGURE 3

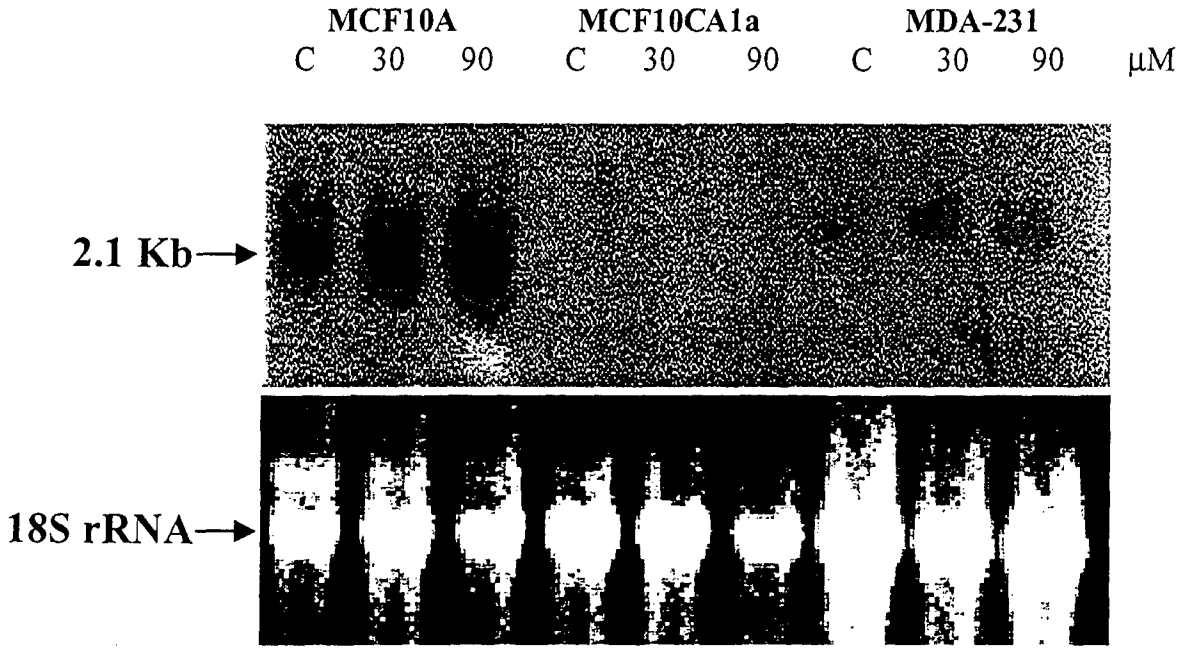


FIGURE 4

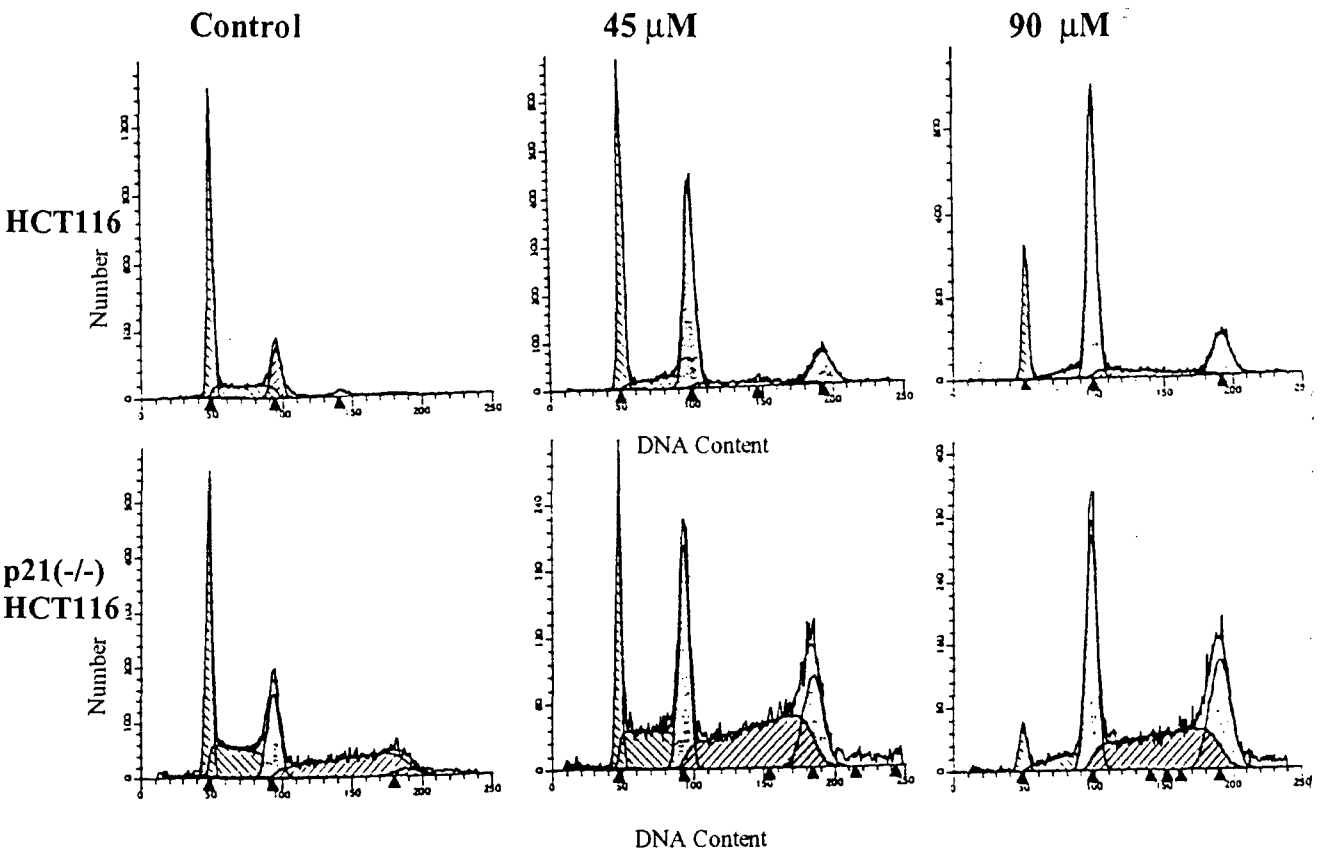

Control
45 μM
90 μM
HCT116
Number
DNA Content
p21(-/-)
HCT116
Number
DNA Content

FIGURE 5

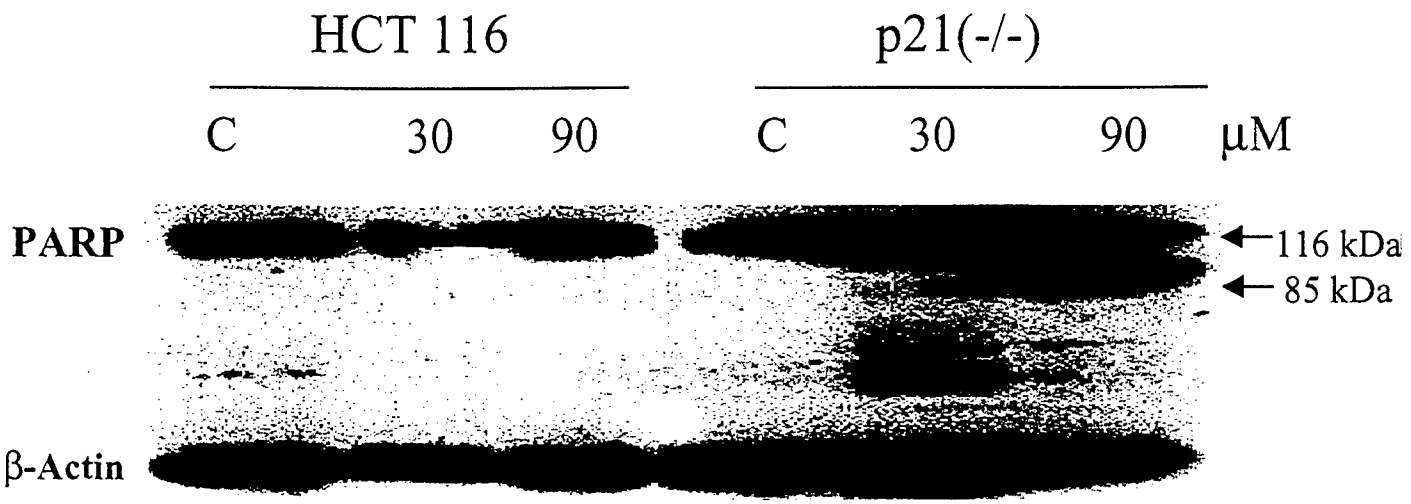
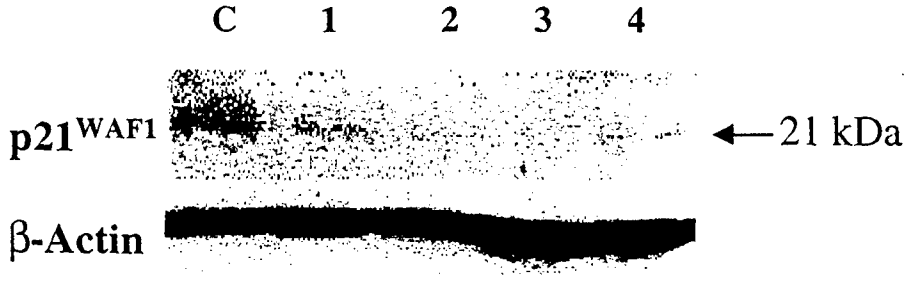


FIGURE 6

MDA-231 $p21^{WAF1}$ ANTI-SENSE CLONES



MCF10A $p21^{WAF1}$ ANTI-SENSE CLONES

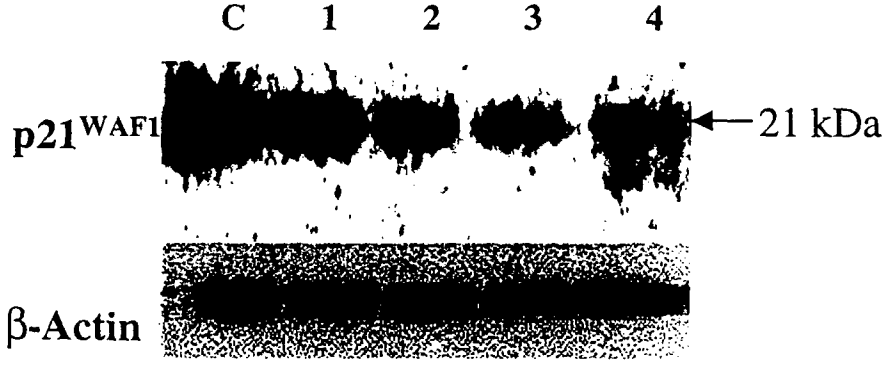


FIGURE 7

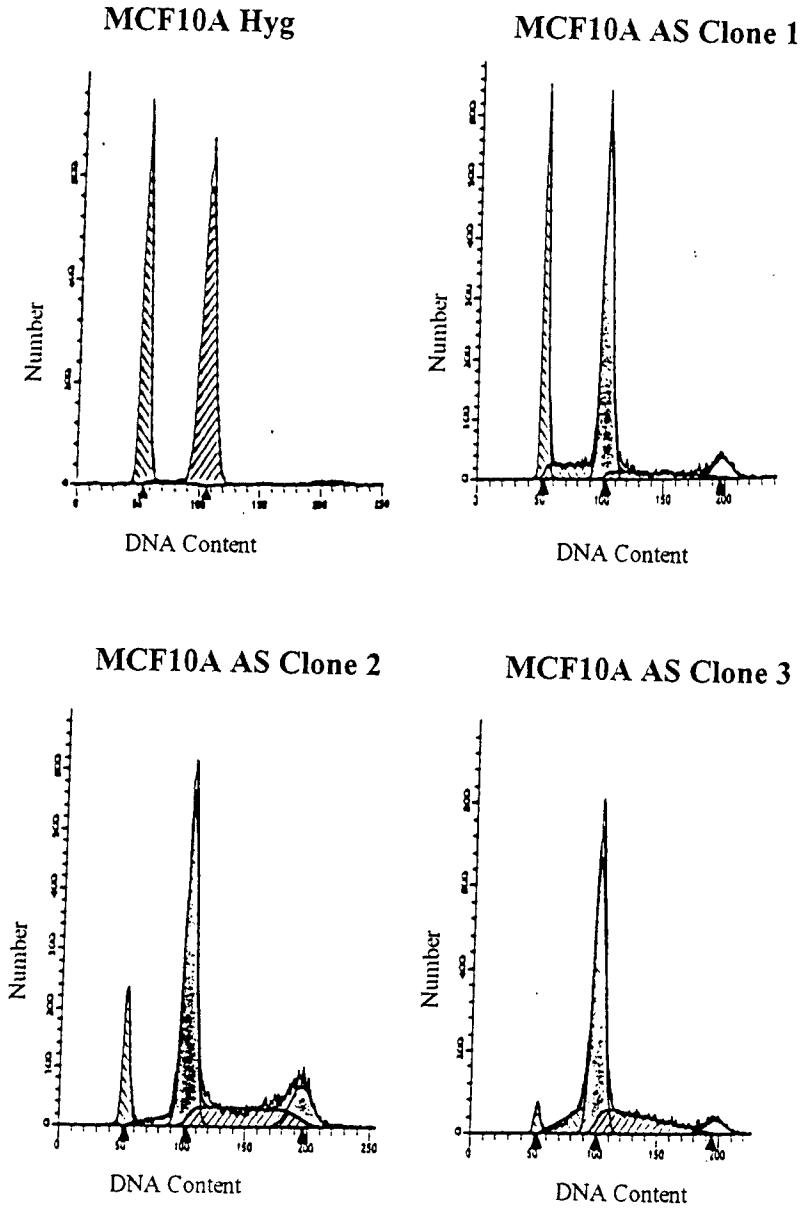
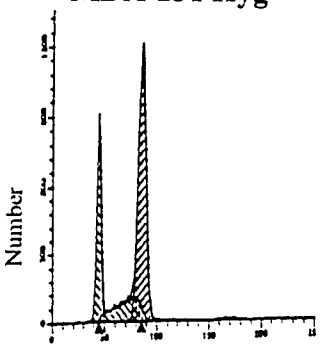
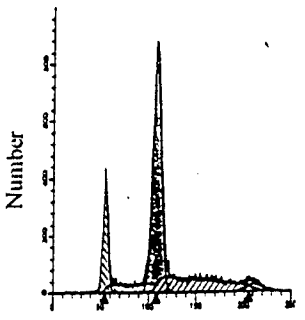

MCF10A Hyg
MCF10A AS Clone 1
MCF10A AS Clone 2
MCF10A AS Clone 3
Number
DNA Content

FIGURE 8

MDA-231 Hyg



MDA-231 AS Clone 2



MDA-231 AS Clone 3

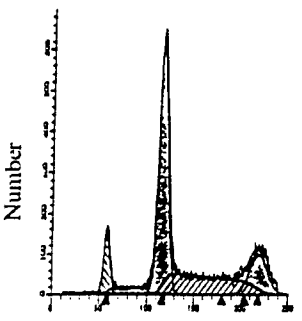


FIGURE 9

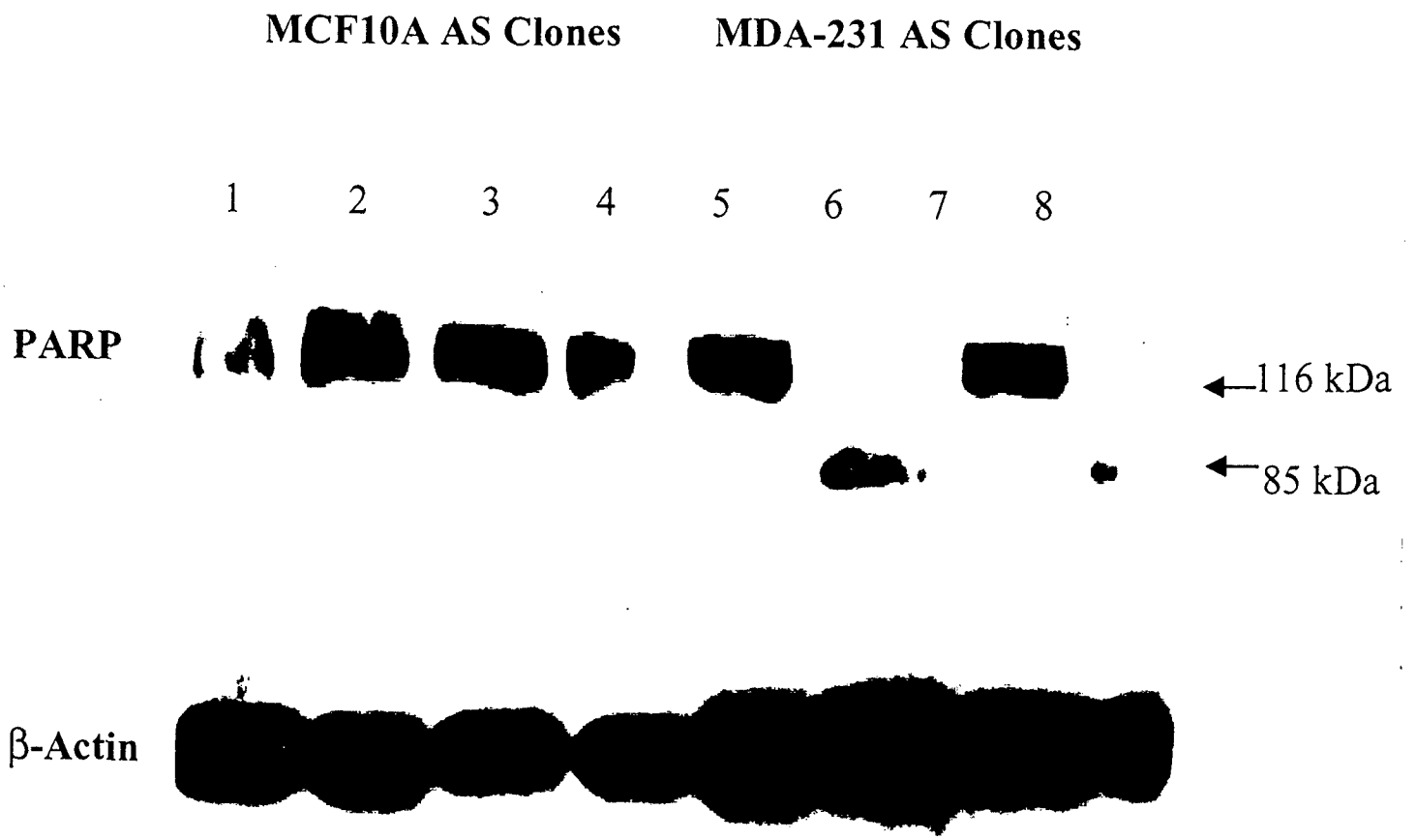

MCF10A AS Clones
MDA-231 AS Clones
1
2
3
4
5
6
7
8
PARP
116 kDa
85 kDa
β-Actin

FIGURE 10

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 27 Sep 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management