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Genistein Inhibits Proliferation Similarly in Estrogen Receptor-Positive and Negative Human Breast Carcinoma Cell Lines Characterized by P21$^{WAF1/CIP1}$ Induction, G_2/M Arrest, and Apoptosis

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Abstract Genistein has been proposed to be responsible for lowering the rate of breast cancer in Asian women but the mechanism for this chemopreventive effect in vivo is unknown. In this study, we present in vitro evidence that genistein inhibits cell proliferation similarly in ER-positive and ER-negative human breast carcinoma cell lines. This inhibition is associated with specific G_2/M arrest and induction of p21$^{WAF1/CIP1}$ expression. Genistein results in a five- to six-fold increase in p21$^{WAF1/CIP1}$ mRNA levels and a three- to four-fold increase in protein levels, only a 1.5-fold increase in p21$^{WAF1/CIP1}$ transcription but a three- to six-fold increase in p21$^{WAF1/CIP1}$ mRNA stability. The increase in p21$^{WAF1/CIP1}$ is followed by increased apoptosis. The similar effects of genistein on a number of breast carcinoma cell lines with different ER and p53 status suggest that the actions of genistein reported here are mediated through ER and p53 independent mechanisms. The chemopreventive effects of genistein in vivo could be mediated along an identical or similar anti-proliferative pathway. J. Cell. Biochem. 69:44–54, 1998. © 1998 Wiley-Liss, Inc.

Key words: genistein; breast cancer; p21$^{WAF1/CIP1}$; G_2/M arrest

Breast cancer chemoprevention is the subject of a substantial effort to improve the health of women in the United States. In epidemiological studies, the consumption of soy isoflavones in Asian women has been linked to decreased rates of breast cancer and it has been suggested that genistein, an isoflavone abundant in soy products, might be responsible for this chemopreventive effect [Parker et al., 1996; Messina et al., 1994; Barnes et al., 1990; Messina and Barnes, 1991].

The mechanism behind genstein's in vivo effects of chemoprevention remains unknown but recently a number of effects of genistein have been observed in vitro. Genistein has been demonstrated to induce differentiation, inhibit topoisomerase II, inhibit angiogenesis and inhibit protein tyrosine kinase [PTK] activity [Barnes and Peterson, 1995; Peterson, 1995; Constantinou and Huberman, 1995; Fotsis et al., 1993]. Because genistein was originally regarded as a naturally occurring estrogen, its effects in human breast cancer were studied initially in ER-positive cells [Peterson and Barnes, 1996; Wang et al., 1996]. Although genistein was originally thought to act through the ER via its angonist/antagonist action in ER-positive breast cancer cell lines [Peterson and Barnes, 1996], subsequent studies in ER-negative lines suggested a different mode of action involving genistein's ability to inhibit PTK [Peterson and Barnes, 1991].

Progression along the cell cycle is tightly regulated and is associated with the sequential activation of a number of genes along key checkpoints [Hartwell and Weinert, 1989]. Orderly flow along the cell cycle requires the function of cyclins, CDKs and CDK inhibitors [Sherr, 1993]. Numerous members of this latter class have now been identified and their role in cell cycle arrest confirmed [Sherr and Roberts, 1995].

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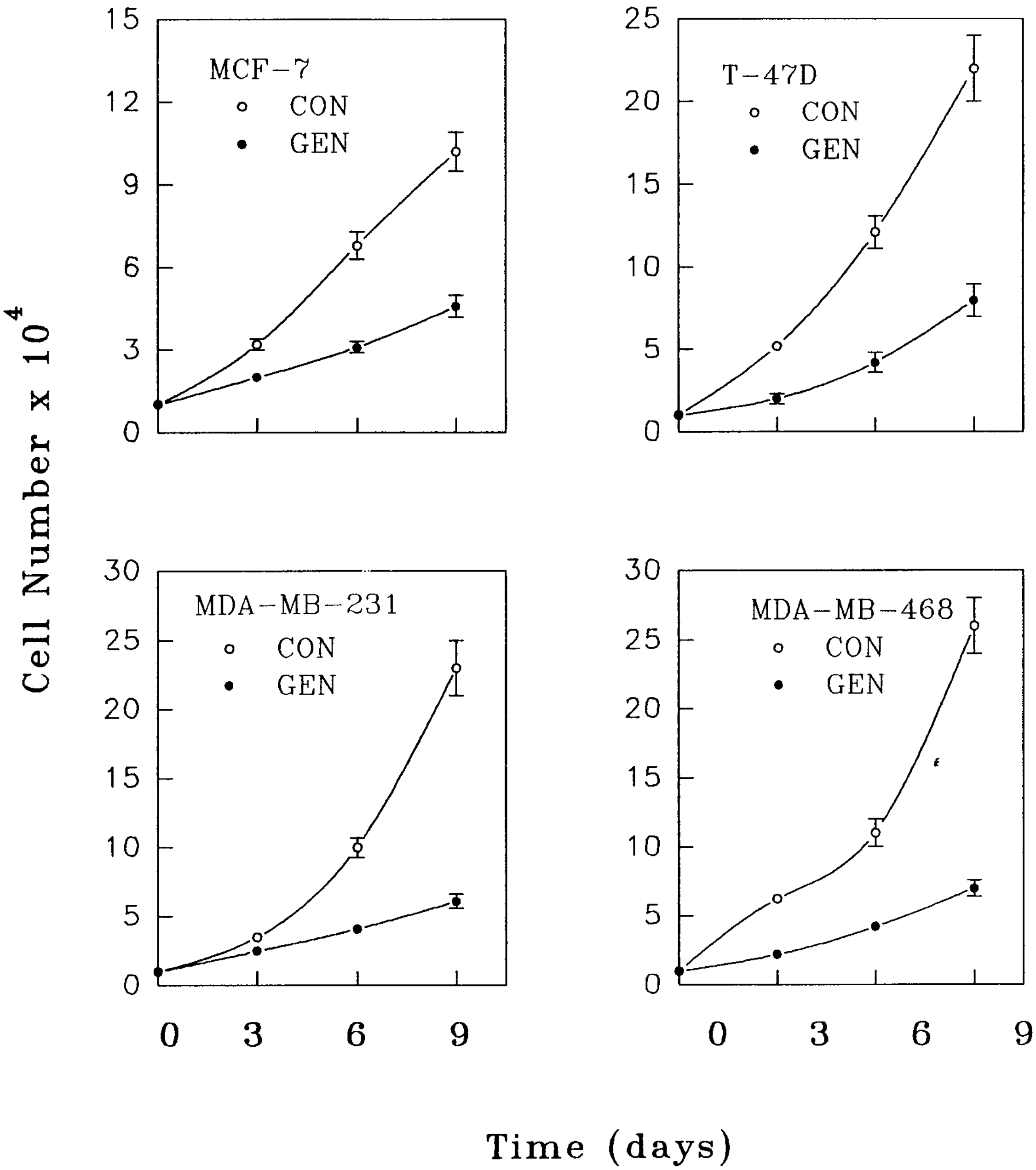

Fig. 1. Genistein inhibition of ER-positive (MCF-7, T47D) and ER-negative (MDA-MB-231, MD-MB-468) breast carcinoma cell proliferation at a dose of 20 µg/ml. The data represent the mean ± SEM of three independent experiments.

These inhibitors have been associated with either CDKs or CDK-cyclin complexes [Sherr and Roberts, 1995; Harper et al., 1993; Xiong et al., 1993]. G_1 arrest of both normal and malignant cells has been associated with the induction of a 21,000 M.W. protein termed $p21^{WAF1/CIP1}$. $p21^{WAF1/CIP1}$ has been found to be a component of cyclin-CDK complexes and is able to modulate the activity of a number of CDKs [Sherr and Roberts, 1995]. Elevation of $p21^{WAF1/CIP1}$ levels enhances $p21^{WAF1/CIP1}$ association with CDK-cyclin complexes, results in decreased CDK activity which in turn inhibits phosphorylation of specific endogenous substrates such as the retinoblastoma protein where inactivation through phosphorylation is required for progression through the cell cycle [Nigg, 1993]. The sustained elevation of $p21^{WAF1/CIP1}$ levels following exposure to DNA-damaging as well as other agents plays a major role in the subsequent G_1

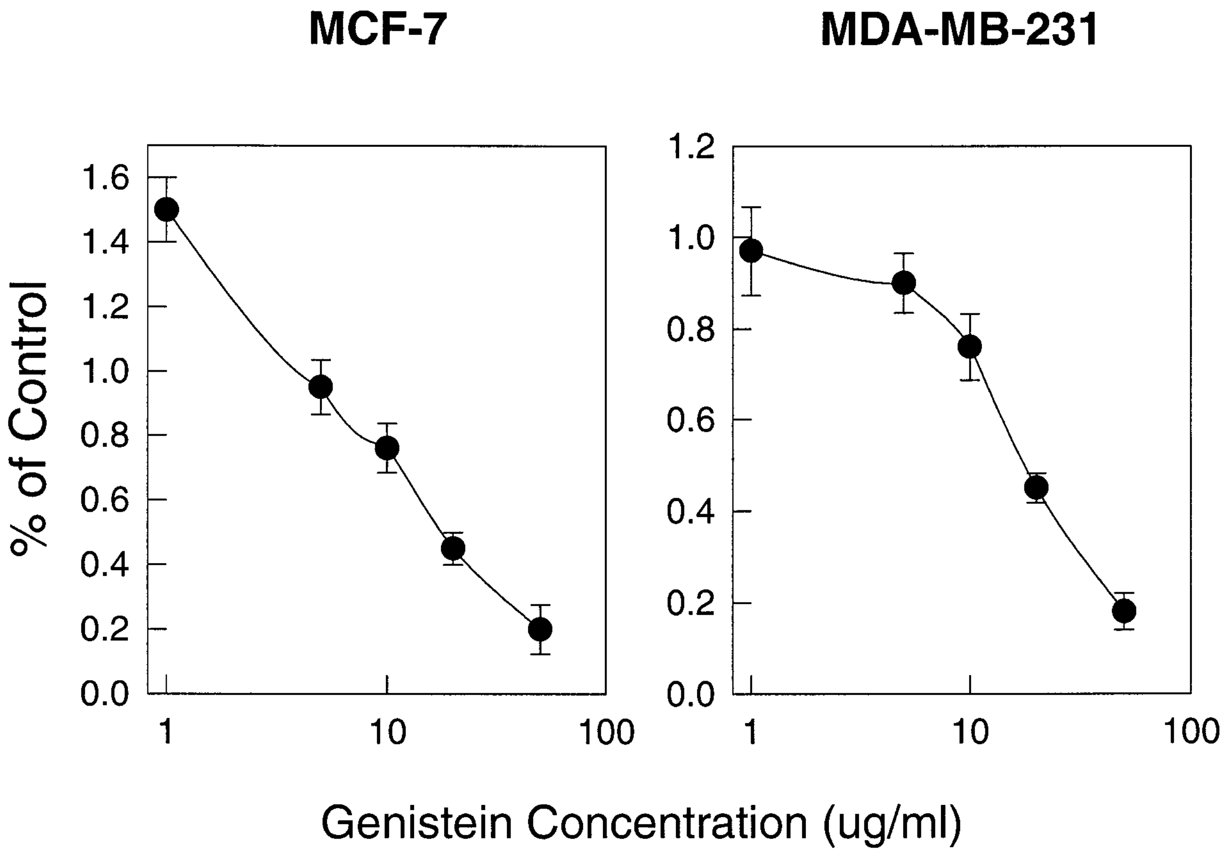

Fig. 2. Effects of varying concentrations of genistein on MCF-7 and MDA-MB-231 cell growth measured after 6 days of treatment. The data represent the mean ± SEM of three independent experiments. Results with T47D and MDA-MB-468 cells were similar.

arrest, inhibition of DNA synthesis, and induction of apoptosis. Transient increased expression of p21$^{WAF1/CIP1}$ also results in inhibition of growth and DNA synthesis [Harper et al., 1993]. Recently it has also been shown that a novel form of p21$^{WAF1/CIP1}$ protein can inhibit growth by acting not at G_1 but rather G_2/M [Tchou et al., 1996].

In the present study, we present experimental evidence suggesting that genistein is able to inhibit cell proliferation similarly in ER-positive and ER-negative human breast carcinoma cell lines. This inhibition is characterized by p21$^{WAF1/CIP1}$ induction, G_2/M arrest and increased apoptosis.

MATERIALS AND METHODS

Reagents

Genistein and propidium iodide were purchased from Sigma Chemical Co. (St Louis, MO). MEM and fetal bovine serum [FBS] were obtained from Life Technologies Inc. GIBCO (Grand Island, NY). [-^{32}P]dCTP (3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). The antiestrogen ICI 182,780 was a gift of Dr. Richard Pietras (Division of Hematology-Oncology, UCLA, Los Angeles, CA).

Cell Lines

The ER-positive MCF-7, T47D and ER-negative MDA-MB-231, MDA-MB-468 cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD). The ER-negative C8161 x MCF-7 hybrid clone recently produced by us by somatic cell fusion [Safarians et al., 1996] was also used. All cell lines were maintained in Eagle MEM with 10% FBS and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin).

Growth Experiments

ER-positive MCF-7, T47D and ER-negative MDA-MB-231, MDA-MB-468 cells were plated at an initial cell concentration of 1 x 10^4 cells per well in MEM medium supplemented with 10% FBS for 24 h and treated with genistein

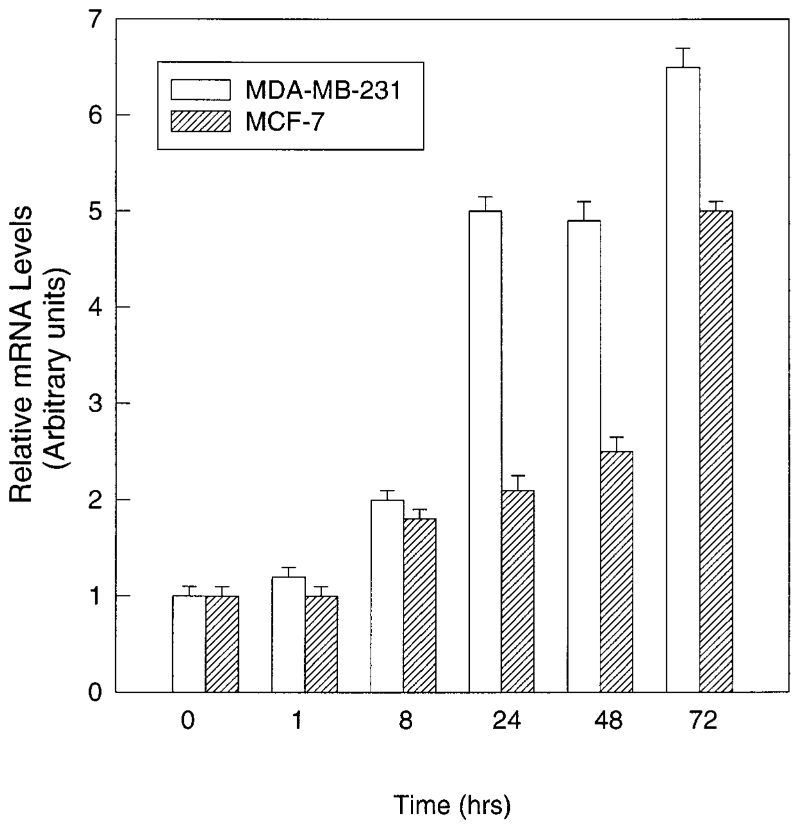

Fig. 3. Genistein induction of $p21^{WAF1/CIP1}$ mRNA expression in MDA-MB-231 and MCF-7 cells. MCF-7 and MDA-MB-231 cells were incubated in MEM supplemented with 10% FBS and genistein (20 µg/ml) was added at various times during a total incubation period of 72 h. Quantitation of Northern blots of three independent experiments are depicted. The values are expressed relative to respective controls which are given arbitrary values of 1. The error bars represent the standard errors. Results with T47D and MDA-MB-468 were similar.

(dissolved in DMSO) for 3, 6, or 9 days for various doses. The final concentration in all the cultures was 0.1%; control cells were incubated with DMSO at the same final concentration.

Plasmid Constructs, Transient Transfection, and Luciferase Assays

Transient transfections were performed as previously described [Shao et al., 1995]. Cells were plated in a 10 cm plate at a density of 3×10^6 cells per plate. The medium was changed 2 h before transfection. The cells were cotransfected with the reporter plasmid and pUC19 (20 µg DNA per plate). Transfection efficiency was normalized with the plasmid pRVSV2 β-galactosidase. Fresh medium and genistein (20 µg/ml) were added following washing the cells with 1X PBS. The cells were harvested 72 h after the transfection. For the luciferase assays, the cells were washed with PBS, and 300–400 µl of lysis buffer (25 mM glycylglycine pH 7.8, 1.5 mM $MgSO_4$, 4 mM EDTA, 100 mM DTT, and 1% Triton X-100) was added to the cells and the cells were harvested. The lysates were centrifuged for 5 min. The supernatants were supplemented with 12 mM K_2HPO4 and 1.6 mM ATP and assayed for luciferase activity by measuring light units in a standard luminometer for 10 s. Relative light units were corrected with respect to β-galactosidase activity. WWP-Luc carries a 2.4 kb 5'-proximal region of the $p21^{WAF1/CIP1}$ gene fused to a promoterless luciferase reporter gene (a gift of Drs. Ken Kinzler and Bert Vogelstein, Johns Hopkins, Baltimore, MD).

Northern and Western blots

Total RNA was extracted and 20 µg of total RNA was loaded in 1.2% agarose gel and Northern blot analysis were performed as previously described [Feinberg and Vogelstein, 1983; Shao et al., 1995]. Studies of $p21^{WAF1/CIP1}$ mRNA stability were performed by incubating cells in the presence or absence of the genistein for 72 hrs followed by the addition of actinomycin-D (4 µg/ml) [Li et al., 1996]. The full-length human

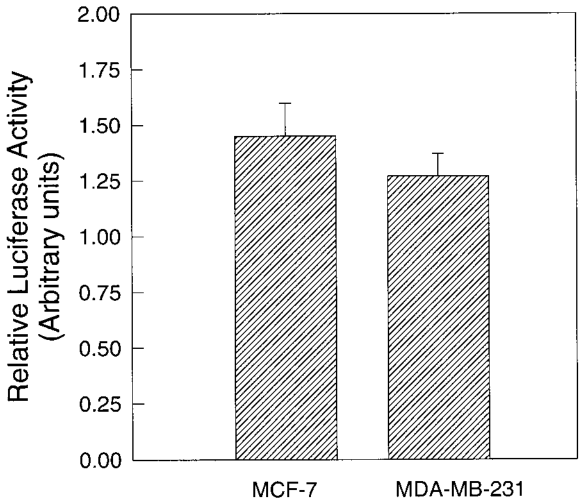

Fig. 4. Genistein-enhanced p21$^{WAF1/CIP1}$ transcription in MCF-7 and MDA-MB-231 cells measured with the p21 WAF1/CIP1 luciferase reporter construct. Results with the other breast carcinoma cell lines were similar.

p21$^{WAF1/CIP1}$ cDNA probe was provided by Drs Kinzler and Vogelstein (Johns Hopkins University, Baltimore, MD). The human cyclin D1 cDNA probes were obtained from Dr. David Beach (Howard Hughes, Cold Spring Harbor Laboratory, Cold Spring, NY). The human cyclin A and B1 cDNA probes were a gift of Dr. Tony Hunter (The Salk Institute, San Diego, CA). Probes were labeled according to the random primer method [Feinberg and Vogelstein, 1983]. Logarithmically growing cells were treated with 20 µg/ml genistein for 0, 1, 8, 24, 48, and 72 h. Western blots using mouse monoclonal p21$^{WAF1/CIP1}$ antibody (Oncogen Research Products, Cambridge, MA) were performed according to standard protocols [Li et al., 1996]. The actions of genistein in the presence of ICI 182,780 (1 µM) [Hyder et al., 1997] were also examined. All cultures were harvested at the end of 72 h incubation period. Bands for both Western and Northern blots were quantitated using a Molecular Dynamics Laser Densitometer (Model PSD1) and an Image Quant Version. 1 software program.

Analysis of Cell Cycle Phase Distribution

Flow cytometry analysis of DNA content was performed to assess the cell cycle phase distribution as described previously [Shao et al., 1995]. Genistein (20 µg/ml) was added to logarithmically growing MCF-7, MDA-MB-231, T47D, and MDA-MB-468 cells. Cells were harvested by trypsinization at various times and stained for DNA content using propidium iodide fluorescence. The computer program Multicycle from Phenix Flow

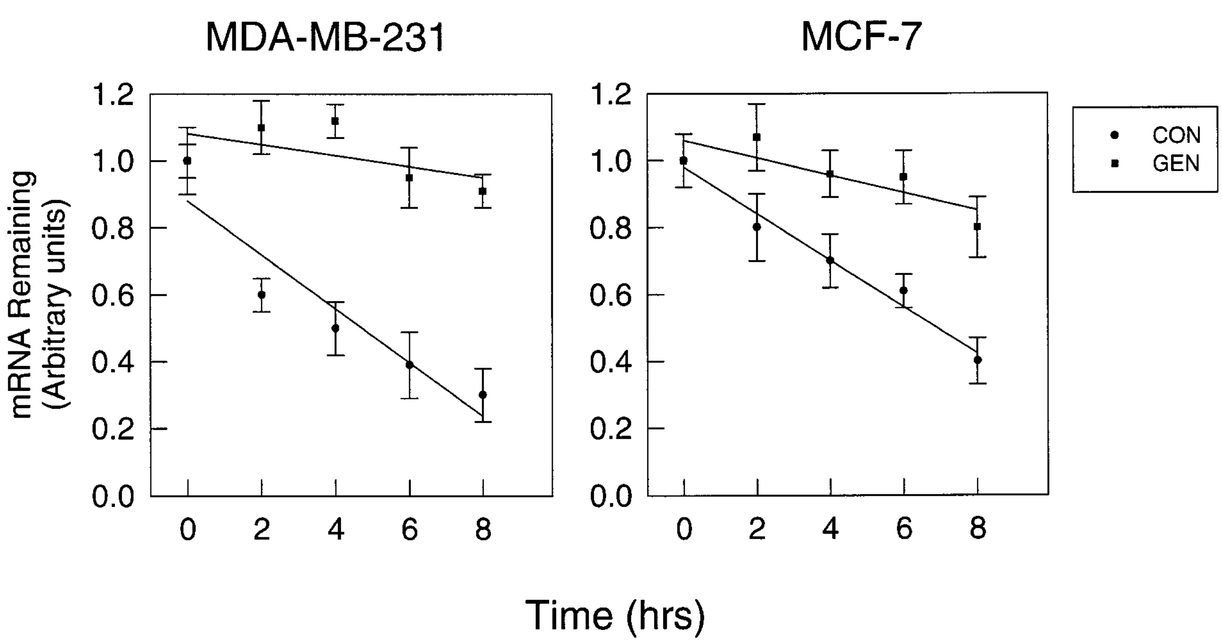

Fig. 5. Effect of genistein on p21$^{WAF1/CIP1}$ mRNA stability in MCF-7 and MDA-MB-231 cells. MCF-7 and MDA-MB-231 cells were incubated with 20 µg/ml of genistein for 72 h. At the end of the treatment, both untreated and treated cells were exposed to 4 µg/ml actinomycin D and then RNA was harvested at certain intervals as indicated, with time 0 defined as the time when actinomycin D was introduced. In the experiment, p21$^{WAF1/CIP1}$ mRNA levels at time 0 in either the absence or presence of genistein are arbitrarily defined as 1. The data represent the mean ±SEM of three independent experiments. Results with the other breast carcinoma cell lines were similar.

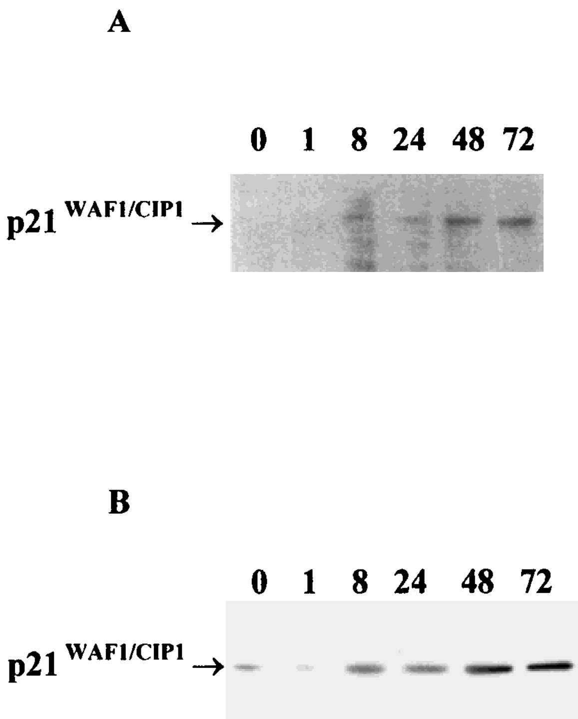

Fig. 6. p21$^{WAF1/CIP1}$protein expression after genistein treatment in **(A)** MCF-7 cells and **(B)** MDA-MB-231 cells. Results with the other breast carcinoma cell lines were similar.

System (San Diego, CA) was used to generate histograms, which were used to determine the cell cycle phase distribution.

Apoptosis Assay

Apoptosis at various time points was detected by labeling the 3'OH ends of DNA utilizing digoxigenin incorporation by terminal deoxynucleotidyl transferase. Antidigoxigenin antibodies and immunoperoxidase staining were utilized to demonstrate digoxigenin-nucleotide incorporation with the ApopTag detection system (Oncor, Gaithersburg, MD). In brief, cells were grown on chamber slides. After fixing with 75% ethanol, slides were rinsed with PBS and incubated in a reaction mixture containing terminal transferase and digoxigenin-UTP at 37°C for 1 h. The specimens were then washed followed by antidigoxigenin antibody coupled to horseradish peroxidase for 30 min at room temperature. After additional washings, diaminobenzidine tetrachloride was added and the cells were incubated for 10 min. The percent peroxidase positive cells were determined by counting 200 cells in random fields in separate experiments.

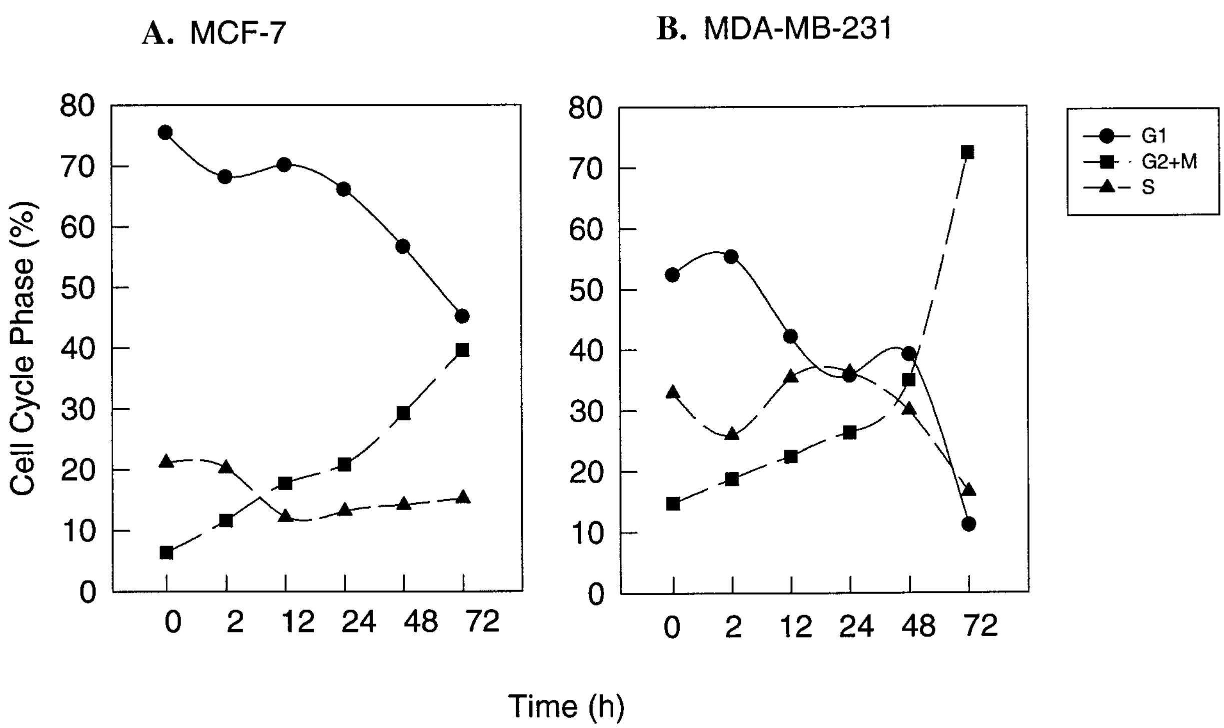

Fig. 7. Effects of genistein (20 µg/ml) on cell cycle progression with varying times of exposure in **(A)** MCF-7 and **(B)** MDA-MB-231 cells. Cell cycle histograms of genistein induction of G_2/M arrest in **(C)** MCF-7 and **(D)** MDA-MB-231 cells at 72 h. A more dramatic G_2/M arrest in MDA-MB-231 cells is in evidence. The MDA-MB-468 line similarly showed a prominent G_2/M peak.

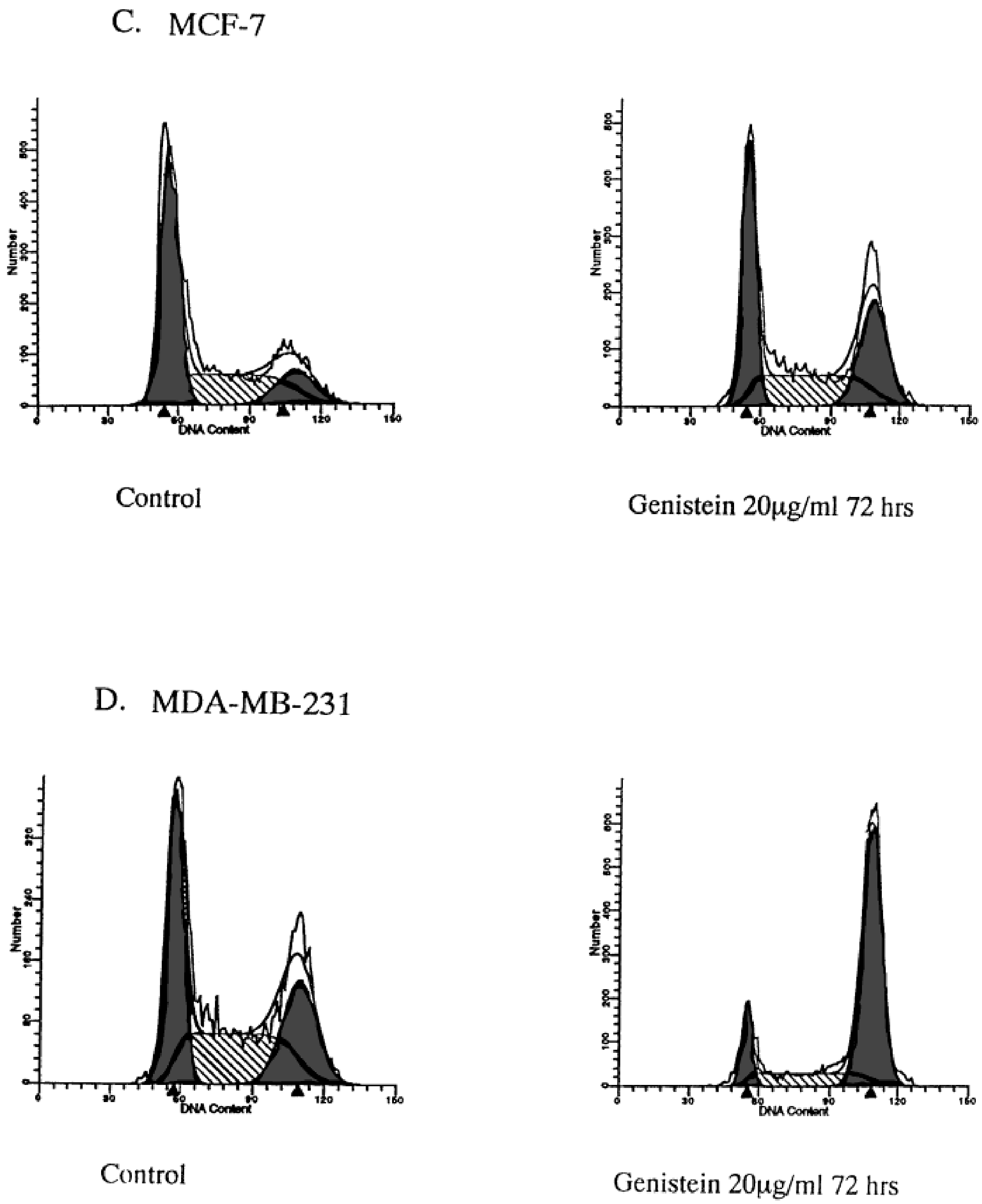

Figure 7. (Continued.)

RESULTS

Genistein Inhibition of ER-Positive and ER-Negative Breast Carcinoma Cell Lines

Genistein inhibited growth of all ER-positive and ER-negative cells similarly (Fig. 1). The concentration of genistein required for 50% inhibition of growth (IC_{50}) for two of these lines, MCF-7 and MDA-MB-231 was 20 μg/ml (Fig. 2). Genistein also similarly inhibited growth of the T47D and the MDA-MB-468 lines as well as the ER-negative somatic cell hybrid produced by fusion of an ER negative line with an ER positive line where ER transcription had been completely abolished.

Genistein Modulation of Cyclin Expression

Genistein induced only modest and bidirectional changes in cyclin expression. Cyclin D1 mRNA levels had little change after 72 h of expo-

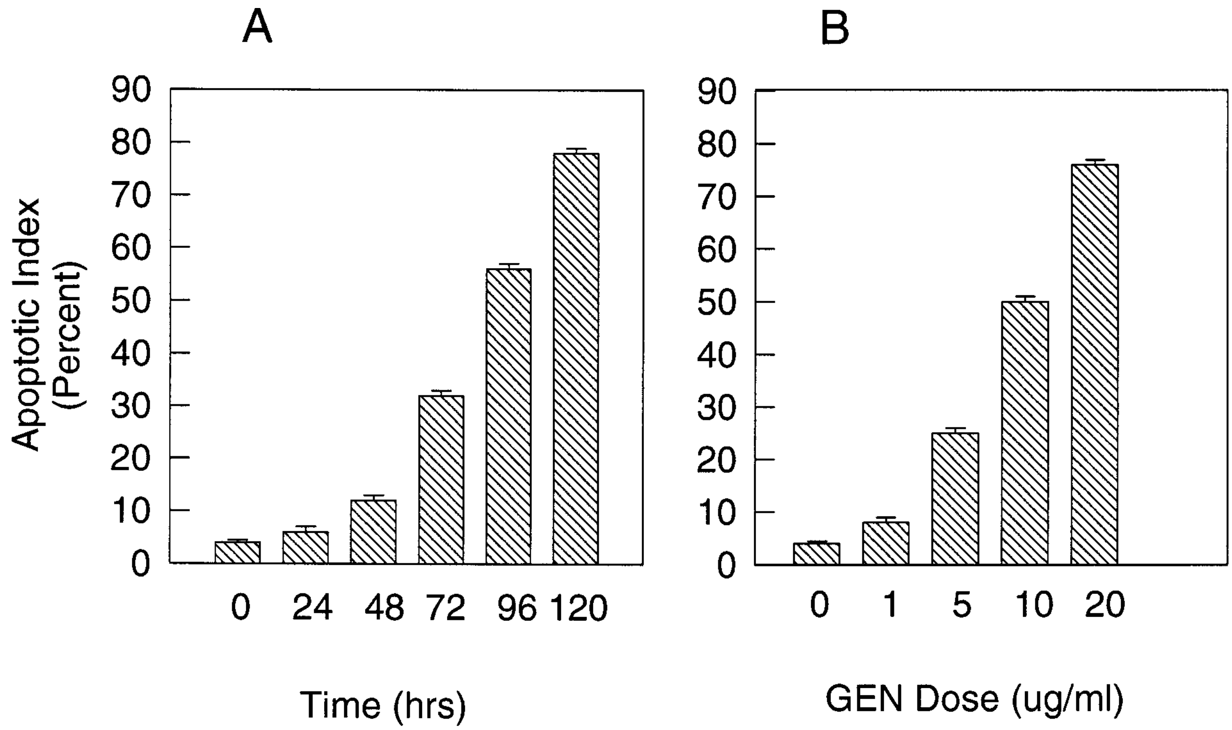

Fig. 8. Genistein-mediated apoptosis in MDA-MB-231 cells with increasing time of exposure **(A)** and genistein concentration **(B)**. In (A), the concentration of genistein was constant at 20 µg/ml; in (B), the time of exposure was constant at 120 h. The results with the other cell lines were similar.

sure to genistein. Cyclin A and B1 mRNA were first noted to decrease after 24 h of exposure then increase two-fold after 48 and 72 h exposure.

Genistein Induction of p21$^{WAF1/CIP1}$ Expression

Exposure of MCF-7, T47D, MDA-MB-231, and MDA-MB-468 cells to 20 µg/ml genistein resulted in the progressive increase in p21$^{WAF1/CIP1}$ mRNA levels with the earliest increase noted at 24 h (Fig. 3). Although exposure of these cells to genistein resulted in an approximately five- to six-fold increase in p21$^{WAF1/CIP1}$ mRNA, respectively (Fig. 3), less than a 1.5-fold increase in p21$^{WAF1/CIP1}$ promoter-mediated gene transcription in both cell lines was noted (Fig. 4). Exposure of these breast carcinoma cell lines to genistein resulted in a three- to six-fold increase in p21$^{WAF1/CIP1}$ mRNA stability in all cell lines (Fig. 5). Predictably these cell lines showed a three- to four-fold increase in p21$^{WAF1/CIP1}$ protein levels respectively at 72 h (Fig. 6). The pure antiestrogen ICI 182,780, in contrast, did not stimulate p21$^{WAF1/CIP1}$ expression in MCF-7 or T47D cells even at its IC_{50} (1 µM). Furthermore ICI 182,780 did not block genistein's stimulation of p21$^{WAF1/CIP1}$ mRNA expression.

Genistein Induction of G_2/M Arrest

No G_1 arrest was observed in genistein-treated cells compared with untreated cells. Rather genistein arrested all of the cell lines in the G_2/M phase as shown by a significant increase in cells in the G_2/M phase of the cell cycle and an accompanied decrease in the percentage in G_1 and S (Fig. 7). The G_2/M arrest was more striking in the ER-negative lines.

Genistein Induction of Apoptosis

Following exposure to genistein (20 µg/ml), all of the breast carcinoma cell lines demonstrated morphological changes of the apoptotic process. The cells showed marked nuclear fragmentation and chromatin condensation with the nuclear and cytoplasmic membranes remaining intact. As indicated by the ApopTag assay, exposure of cells to genistein resulted in a significant amount of apoptosis at 72 h in all of the cell lines. Also increasing genistein concentrations resulted in increased apoptosis. Exposure to 20 µg/ml of genistein for 120 h resulted in 80% of the cells in all of the cell lines demonstrating apoptosis (Fig. 8).

DISCUSSION

Genistein has been shown to inhibit proliferation of a number of human cancer cells including breast cancer cells [Messina et al., 1994; Peterson and Barnes, 1991, 1996; Wang et al., 1996]. Several mechanisms have been proposed for the effects of genistein. Initially, genistein was considered to function as an estrogen receptor agonist/antagonist [Peterson and Barnes, 1996]. Later several other mechanisms were proposed including topoisomerase II inhibition, induction of differentiation, induction of oxidation events and PTK inhibition [Barnes and Peterson, 1995; Peterson, 1995; Constantinou and Huberman, 1995; Fotsis et al., 1993]. In the present study, we propose another mechanism by which genistein inhibits cell proliferation in both ER-positive and ER-negative cells, G_2/M arrest characterized by $p21^{WAF1/CIP1}$ expression with subsequent apoptosis. $p21^{WAF1/CIP1}$ has been demonstrated to play a crucial role in G_1 checkpoint and appears to be required for G_1 arrest following DNA damage [Hartwell and Weinert, 1989; O'Conner and Kohn, 1992]. $p21^{WAF1/CIP1}$ has also been implicated in G_1 arrest in response to irradiation by inhibiting $p33^{CDK2}$/cyclin E protein complexes [Levedakou et al., 1995]. Expression of $p21^{WAF1/CIP1}$ fluctuates throughout the cell cycle, but peaks at G_1 and G_2 and is tightly regulated [Levedakou et al., 1995]. In our study, genistein was seen to act on G_2/M and not G_1. The findings of only minimal and bidirectional alterations in cyclins D, A1, and B1, cyclins which are thought to regulate progression through G_1 into S, also indicates that genistein was not acting on G_1. Recently it has been shown that 12-0-tetradecanoylphorbol-13-acetate [TPA] can activate a protein kinase C [PKC]-mediated, negative-growth regulatory pathway leading to the induction of expression of $p21^{WAF1/CIP1}$ which mediates G_2/M arrest [Tchou et al., 1996]. In that study however, the $p21^{WAF1/CIP1}$ protein responsible for G_2/M arrest was a novel protein formed by posttranslational modification of $p21^{WAF1/CIP1}$ whose C-terminal region was truncated [Tchou et al., 1996]. In our study, we found no evidence for this truncated protein but instead observed the wild type $p21^{WAF1/CIP1}$ and G_2/M arrest. Although p21 is a gene that acts downstream of p53, p53-independent regulation of $p21^{WAF1/CIP1}$ expression has been demonstrated in a number of systems [Shao et al., 1995; Li et al., 1994; Agarwal et al., 1995; Michieli et al., 1994]. Also several potential mechanisms involved in p53-independent activation of $p21^{WAF1/CIP1}$ expression have now been reported [Batto et al., 1995]. The addition of TGFβ to HaCaT cells that possessed mutant p53 alleles resulted in the enhanced transcription of the $p21^{WAF1/CIP1}$ gene at the activation of an element in the $p21^{WAF1/CIP1}$ promoter distinct from the p53 consensus sequence [Batto et al., 1995]. El-Deiry et al. [1995] have identified a response element in the $p21^{WAF1/CIP1}$ promoter that could play a role in p53-independent enhancement of transcription of the $p21^{WAF1/CIP1}$ gene. p53-independent regulation of $p21^{WAF1/CIP1}$ has been found to occur at multiple levels. Increase in the translation of $p21^{WAF1/CIP1}$ message in breast carcinoma cells following DNA damage induced by radiation has been found, suggesting that translational regulation plays an important role in the regulation of $p21^{WAF1/CIP1}$ expression in these cells [Gudas et al., 1995]. In our study, we found that the p53-independent increase in $p21^{WAF1/CIP1}$ occurred predominately through increased mRNA stability.

Genistein similarly and significantly inhibited proliferation of both ER-positive (MCF-7 and T47D) and ER-negative (MDA-MB-231 and MDA-MB-468) lines in part by causing a G_2/M arrest. This G_2/M arrest however was more striking in the ER-negative lines. The reasons for this are not clear at the present time. Since the ER-negative lines also contain abnormal p53, it may well be that the effects of genistein in inducing a G_2/M arrest are not totally independent of ER and p53 status. Although it has been reported that genistein causes G_2/M arrest in leukemic and human gastric carcinoma cells [Matsukawa et al., 1993; Traganos et al., 1992], these studies have not addressed the role of p21. Since most of the time p21 is associated with G_1 arrest, the G_2/M arrest noted here may be specifically related to a unique genistein $p21^{WAF1/CIP1}$ pathway. This G_2/M arrest was prominent at 72 h and was preceded by a significant increased $p21^{WAF1/CIP1}$ mRNA and protein levels at 48 h. Since MDA-MB-231 cells possess both mutant (nonfunctional) p53 [Sheikh et al., 1994; Niewolik et al., 1995] and lack of ER, the genistein-mediated pathway leading to increased $p21^{WAF1/CIP1}$ in this cell line is a p53 and ER independent pathway. Furthermore the lack of effect of ICI 182,780 in blocking genistein's action in MCF-7 cells also supports an ER independent pathway in this second cell line as well.

Although exposure of MCF-7 and MDA-MB-231 cells to genistein resulted in a five- and six-fold increase in $p21^{WAF1/CIP1}$ mRNA respectively, this was accompanied by less than a 1.5-fold increase in transcription. The modulation of $p21^{WAF1/CIP1}$ mRNA expression at a post-transcriptional level was indicated by three- to six-fold increases in $p21^{WAF1/CIP1}$ message stability as noted. The induction of p21 mRNA stability is not unique to genistein but has been found with other agents such as retinoids [Li et al., 1996].

Exposure of breast carcinoma cells to genistein resulted in the onset of apoptosis, which appeared to follow $p21^{WAF1/CIP1}$ induction and G_2/M arrest. Genistein has been observed in other studies to induce apoptosis of other cell lines. For example it has been shown that genistein induces apoptosis in Jurkat T-leukemia cells [Spinozzi et al., 1994]. It has also been recently reported that over-expression of $p21^{WAF1/CIP1}$ in human breast carcinoma cells results in apoptosis [Sheikh et al., 1995]. Whether genistein-induced apoptosis is trigured by $p21^{WAF1/CIP1}$ levels remains to be determined.

In summary, our findings demonstrate that genistein has a strong inhibitory effect on cell proliferation which is similar in both ER-positive and ER-negative human breast cancer cells. This inhibitory effect on cell proliferation is at least partially the result of G_2/M arrest and is accompanied by increased $p21^{WAF1/CIP1}$ expression, mediated in large part by stabilizing effects on p21 mRNA. In this study we have not shown that $p21^{WAF1/CIP1}$ expression is causally related to the effects of genistein treatment. To demonstrate this relationship, functional studies with antisense p21 constructs are in progress. Genistein also induced apoptosis in all of the breast carcinoma lines studied. All of these anti-proliferative effects of genistein were largely independent of ER and p53. The chemopreventive effects of genistein in vivo could be mediated along an identical or similar anti-proliferative pathway.

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