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Effects of genistein on cell proliferation and cell cycle arrest in nonneoplastic human mammary epithelial cells: involvement of Cdc2, $p21^{waf/cip1}$, $p27^{kip1}$, and Cdc25C expression

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Abstract

Genistein, a soy isoflavone, has been reported to inhibit the multiplication of numerous neoplastic cells, including those in the breast. However, there is limited information on the effect of genistein on nonneoplastic human breast cells. In the present studies, genistein inhibited proliferation of, and DNA synthesis in, the nonneoplastic human mammary epithelial cell line MCF-10F with an IC_{50} of ~19–22 μM, and caused a reversible G2/M block in cell cycle progression. Genistein treatment (45 μM) increased the phosphorylation of Cdc2 by 3-fold, decreased the activity of Cdc2 by 70% after 8 hr, and by 24 hr reduced the expression of Cdc2 by 70%. In addition, genistein enhanced the expression of the cell cycle inhibitor $p21^{waf/cip1}$ by 10- to 15-fold, increased $p21^{waf/cip1}$ association with Cdc2 by 2-fold, and increased the expression of the tumor suppressor p53 by 2.8-fold. Genistein did not alter the expression of $p27^{kip1}$ significantly. Furthermore, genistein inhibited the expression of the cell cycle-associated phosphatase Cdc25C by 80%. From these results, we conclude that genistein inhibits the growth of nonneoplastic MCF-10F human breast cells by preventing the G2/M phase transition, induces the expression of the cell cycle inhibitor $p21^{waf/cip1}$ as well as its interaction with Cdc2, and inhibits the activity of Cdc2 in a phosphorylation-related manner. Down-regulation of the cell cycle-associated phosphatase Cdc25C combined with up-regulation of $p21^{waf/cip1}$ expression appear to be important mechanisms by which genistein decreases Cdc2 kinase activity and causes G2 cell cycle arrest.

Keywords: Genistein; Cdc2; $p21^{waf/cip1}$; $p27^{kip1}$; Cdc25C; MCF-10F; Cell cycle

1. Introduction

Phytoestrogens present in legumes and other plants have been the subject of considerable attention due to their reported beneficial impact on chronic diseases including breast cancer [1,2]. The phytoestrogen isoflavones present in soybeans, in particular, have a broad range of properties that may contribute to their cancer chemoprotective action. The cancer protective actions of these compounds, of which genistein is one of the most active, include antiestrogenicity, inhibition of oxidative stress, suppression of cell proliferation, induction of differentiation, and modulation of apoptosis [3–5]. Genistein can inhibit multiplication of transformed cells from numerous tissues and can retard tumorigenesis in experimental cancer models [4–7]. The biochemical mechanisms behind the effects of genistein have been reported to include its interaction with the estrogen receptor, its action as an antioxidant, inhibition of topoisomerase II, and inhibition of PTK activities [8–14]. These events generally are associated with a G2/M block in cell cycle progression [15–18], although some cell types can respond to genistein by a block at G1 [19,20]. For breast cancer cells specifically, the antiproliferative action of genistein is not dependent on ER expression or on a broad inhibition of PTK activity [21–24]. Additionally, nontransformed breast cells have been reported to be more sensitive than transformed cells to the antiproliferative action of genistein [25]. This difference in inhibitory action of genistein is due, in part, to differences in isoflavone metabolism based on transformation status. The present studies

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Abbreviations: PTK, protein tyrosine kinase; cdk, cyclin-dependent kinase; DMEM, Dulbecco's Modified Eagle's Medium; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; and ER, estrogen receptor.

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were initiated in order to characterize the cell cycle-associated antiproliferative effects of genistein on the ER-negative nonneoplastic MCF-10F human breast epithelial cell line [26]. In particular, we have chosen to examine the impact of genistein on Cdc2-associated events accompanying the transition of cells from the G2 to M stages of the cell cycle [27].

The progression of eukaryotic cells from the G2 to M phase of the cell cycle depends on the activity of a functional complex formed between cyclin B1 and the cdk Cdc2 [27]. Cyclin B1 expression is increased as cells enter the G2 phase of the cell cycle, with cyclin B1 immediately binding to the cdk Cdc2 [28]. In addition to the content of cyclin B1, progression to mitosis can be affected by the phosphorylation status of Cdc2. Mitotic entry can be delayed by phosphorylation of Cdc2 at Tyr-15 by Wee1 kinase in the ATP binding domain [29]. On the other hand, dephosphorylation of Tyr-15 by the phosphatase Cdc25C [30] increases the kinase activity of the cyclin B1–Cdc2 complex in the M phase. Cdc25C expression has been demonstrated to be down-regulated by genistein in HN4 squamous carcinoma cells and is an indicator of G2/M arrest and the anti-proliferation effects of genistein [31]. As cells enter the M phase, cyclin B1–Cdc2 complexes are translocated from the cytoplasm to the nucleus [32]. The activity of the complexes is also affected by the content of cdk inhibitors [32]. In MDA-MB-231 breast cancer cells, the inhibition of G2 progression is correlated with increased contents of total $p21^{waf/cip1}$ and of $p21^{waf/cip1}$–cyclin A complexes and with an inhibition of cyclin A- and B1-associated histone H1 kinase activities [33]. In MCF-7 breast cancer cells, $p21^{waf/cip1}$ induced G2/M cell cycle arrest independently of its G1 arrest [16]. In aphidicolin-synchronized MCF-7 cells, cell-cycle arrest occurred in parallel with the inhibition of Cdc2 activity [34]. In addition, phorbol 12-myristate 13-acetate (PMA)-induced protein kinase C enhanced the associations between $p21^{waf/cip1}$ and either cyclin B or Cdc2 [34]. Furthermore, $p21^{waf/cip1}$ was able to associate with the active Tyr-15 dephosphorylated form of Cdc2. However, this complex was devoid of kinase activity, indicating that $p21^{waf/cip1}$ may play a role in the inhibition of Cdc2 and G2 arrest [34]. Although there has been much study of the action of genistein in neoplastic human cells, there is far less known about its actions in nonneoplastic tissue, particularly the breast. This is important, since there is a growing interest in recommending natural products, including genistein-enriched soy extracts, for health promotion and cancer prevention among healthy women. The aims of the present study, therefore, were to characterize the antiproliferative action of genistein in nonneoplastic human breast epithelial cells and to examine the effectiveness of genistein in modulating the activity and phosphorylation status of Cdc2, the expression of the cdk inhibitors $p21^{waf/cip1}$ and $p27^{kip1}$, and the expression of the phosphatase Cdc25C.

2. Materials and methods

2.1. Materials

Horseradish peroxidase-conjugated secondary antibodies, $p21^{waf/cip1}$ (Cat. No. SC 397), $p27^{kip1}$ (Cat. No. SC 528), Cdc25C (Cat. No. SC 327), non-phospho-specific Cdc2 antibodies (Cat. No. SC 54), and protein A-Sepharose were obtained from Santa Cruz Biotechnology. Phospho-specific Cdc2 antibody (Cat. No. 9111) was obtained from New England Biolabs. Anti-p53 sheep serum (Cat. No. PC35) and rabbit anti-sheep biotinylated secondary antibodies were obtained from Oncogene Research Products. Histone H1 was obtained from Calbiochem. Genistein was purchased from the Indofine Chemical Co., Inc., and MTT from the Sigma Chemical Co. MCF-10F cells were obtained from the Michigan Cancer Foundation. Growth media and serum were obtained from GIBCO BRL. [^{3}H]Thymidine and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from NEN, and ECL reagents from Amersham.

2.2. Cell culture conditions

MCF-10F cells were grown in DMEM/F12 medium supplemented with 5% horse serum, 0.5 μg/mL of hydrocortisone, 0.1 μg/mL of cholera toxin, 10 μg/mL of insulin, 0.02 μg/mL of epidermal growth factor (EGF) and 100 IU/mL of penicillin-streptomycin in an atmosphere of 5% CO_2/95% air at 37°.

2.3. MTT assay and [^{3}H]thymidine incorporation

The inhibition of cell proliferation by genistein was determined by use of the MTT assay [35], which monitors the numbers of cells based on the reduction of MTT by the mitochondrial dehydrogenases present in viable cells, and by the incorporation of [^{3}H]thymidine into multiplying cells. For the MTT assay, cells were plated into 96-well tissue culture dishes at a density of 1×10^3 cells/well in 200 μL medium. After plating, the cells were allowed to attach for 2 days. Genistein was added using DMSO as the vehicle, with the maximum concentration of DMSO being 0.1%. Incubation with genistein continued for 4 days, at which time 50 μL of 2 mg/mL of MTT was added, and the absorbance at 550 nm was determined by a microtiter plate reader. The absorbance at 690 nm was also measured to compensate for interfering effects of cell debris and the microtiter plate. Experiments were conducted in triplicate.

For the measurement of [^{3}H]thymidine incorporation, MCF-10F cells were plated at 1×10^4 cells/cm^2 in 24-well dishes. Two days later, when the cells were in the exponential growth phase, the control medium was changed to medium containing genistein or DMSO vehicle. Cells were incubated with genistein or vehicle for 24 hr, and [^{3}H]thymidine incorporation was determined as previously described [36].

2.4. Cell cycle analysis

Asynchronous cultures of MCF-10F cells were grown in T-75 flasks to 20–30% confluency, at which time the culture medium was replaced and an aliquot of genistein in DMSO added. After the allotted periods of exposure to genistein, cells were harvested by trypsin release and resuspended in modified Vindelov's DNA staining solution (10 mg/mL of RNase A, 0.1% NP-40, 8.5 mg/mL of propidium iodide, in PBS) at a density of $\sim 10^6$ cells/mL. Within 1–2 hr, the fluorescence of the cells was measured (at rates of 10,000/sec) with a Coulter® EPICS 750 series flow cytometer. Data analysis was performed by Coulter® EPICS XL/XL-MCL, System II (TM) software.

2.5. Western blotting

Asynchronous cultures of MCF-10F cells were plated in control medium and 2 days later changed to medium containing genistein or DMSO vehicle for 2–36 hr, at which time cells were harvested as described previously [36–38]. Briefly, cells were lysed in lysis buffer (1% sodium deoxycholate; 1% Triton X-100; 0.01% SDS; 150 mM NaCl; 50 mM Tris, pH 7.5; 0.5 mM EDTA; 50 mM NaF; 10 mM $NaPP_i$; 0.5 mM Na_3VO_4; 1 mM phenylmethylsulfonyl fluoride, and 20 μg/mL of aprotinin, leupeptin, and pepstatin) and sonicated for 10 sec. Cell lysates were centrifuged at 14,000 *g* at 4° for 5 min. Supernatants were collected, and total protein concentrations were determined. Equal amounts of protein were separated by SDS–PAGE (7.5% polyacrylamide) and transferred to nitrocellulose. The Tyr-15 phospho-Cdc2 protein was detected by immunoblotting the nitrocellulose membrane with a rabbit polyclonal phospho-specific Cdc2 (1:1000 dilution) antibody, overnight at 4°. All other primary antibodies were used at a concentration of 1 mg/mL and incubated for 1 hr at 25°. Membranes were then incubated with a rabbit IgG horseradish peroxidase conjugate for 1 hr at 25°. Proteins were visualized by enhanced chemiluminescence.

2.6. In vitro *Cdc2 histone H1 kinase assay*

MCF-10F cells were plated as described for thymidine incorporation and western blotting. Histone H1 kinase activity was assayed in Cdc2 immune complexes isolated from cells treated with genistein or DMSO vehicle for 8–24 hr. Cells were lysed in ice-cold lysis buffer (20 mM Tris, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 2.5 mM sodium pyrophosphate; 1 mM β-glycerol-phosphate; 1 mM Na_3VO_4; and 1 μg/mL of leupeptin). Cell lysates were sonicated for 10 sec and centrifuged at 14,000 *g* for 5 min. Supernatants were precleared by rotation with protein A-Sepharose for 30 min at 4°. Total cell lysates (300 μg protein/sample) were rotated with 2 μg anti-Cdc2 antibody for 1 hr at 4°. Immune complexes were collected on protein A-Sepharose beads and washed two time with lysis buffer and twice with kinase assay buffer (25 mM Tris, pH 7.5; 5 mM β-glycerol-phosphate; 2 mM dithiothreitol; 0.1 mM Na_3VO_4; 10 mM $MgCl_2$), resuspended in 50 μL of kinase buffer containing ATP (5 μM), histone H1 (5 μg), and [γ-^{32}P]ATP (5 μCi, 3000 Ci/mmol), and incubated for 30 min at 30°. Reactions were stopped by the addition of 50 μL of sample buffer. Samples were then boiled for 5 min and analyzed on SDS–PAGE (7.5% polyacrylamide), and transferred to nitrocellulose. Quantitation of radiolabeled histone H1 was determined by autoradiography of gels and nitrocellulose with densitometry.

2.7. Immunoprecipitation/immunoblot

For association of $p21^{waf/cip1}$ with Cdc2, precleared cell lysates (300 μg) were incubated with 10 μL anti-Cdc2 for 1 hr at 4°. Immunocomplexes were resolved by 7.5% SDS–PAGE and were transferred to nitrocellulose. Association of $p21^{waf/cip1}$ with Cdc2 was detected by incubating the blots with anti-$p21^{waf/cip1}$ as described above.

2.8. Statistical methods

Data are expressed as means ± SD. Statistical differences were analyzed using Student's paired *t*-test. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Effect of genistein on cell proliferation and cell cycle progression

Previous reports have demonstrated that genistein is a potent inhibitor of human breast cancer cell proliferation [4,5,8,16]. However, the relationship between genistein inhibition of cell proliferation and Cdc2 activity has not been examined in nonneoplastic human mammary epithelial cells. Incubation of MCF-10F cells with 45 μM genistein resulted in inhibition of the growth of MCF-10F cells by $\sim$90%, with an IC_{50} value of approximately 19–22 μM (Fig. 1A). As determined by [^{3}H]thymidine incorporation, DNA synthesis at concentrations of genistein as low as 0.1, 0.5, 1, 5, and 10 μM was (mean ± SD) 87.2 ± 5.1, 82.3 ± 10.3, 82.5 ± 8.5, 78.9 ± 2.1, and 71.9 ± 5.5% of controls, respectively (Fig. 1B). At a genistein concentration of 45 μM, DNA synthesis was inhibited by 90% (Fig. 1B). Phase contrast microscopy did not reveal any morphological changes or characteristics of apoptosis such as membrane blebbing and formation of apoptotic bodies after treatment with genistein (data not shown).

With regard to the effect of genistein on cell cycle progression, incubation with genistein (30 μM) caused an increase in the number of MCF-10F cells present in the G2/M phase of the cell cycle (Fig. 2). A significant G2 block was

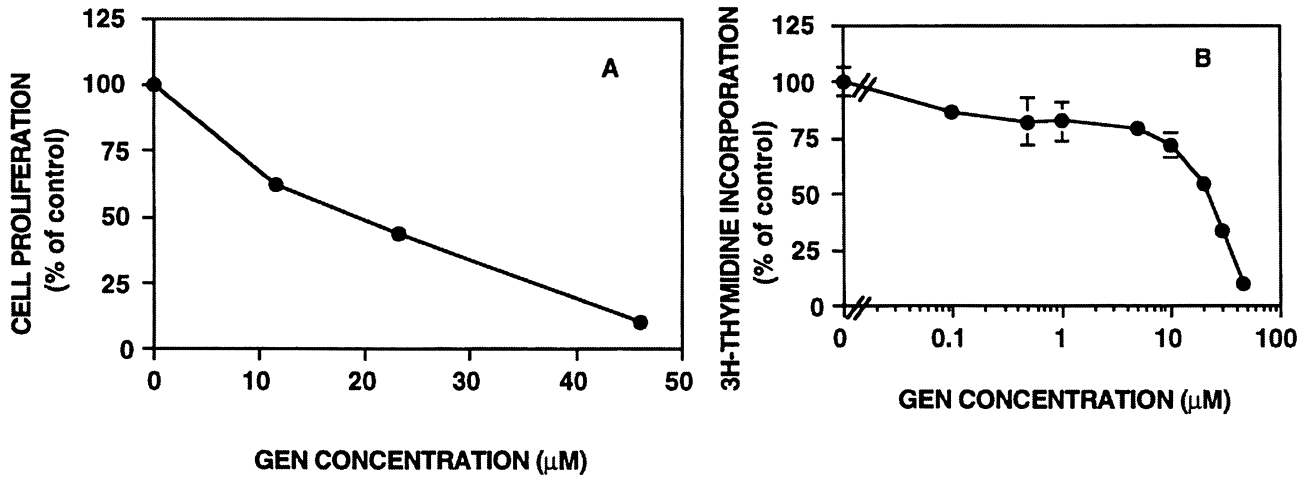

Fig. 1. Concentration-dependent effect of genistein on MCF-10F cell proliferation as determined by MTT assay (A) or [^{3}H]thymidine incorporation (B). Data points in (A) represent means of two experiments, and in (B) means ± SD (N = 3). The 100% value in (B) represents 569 dpm.

evident after a 36-hr but not a 24-hr incubation of MCF-10F cells with genistein at concentrations of 15 and 30 μM (data not shown).

To determine whether the inhibition by genistein of G2 to M progression in MCF-10F cells was transient or permanent, cells were incubated with 45 μM genistein for 48 hr and then transferred to medium without genistein. Within 48 hr of removal of genistein from the medium, cell cycle distribution returned to that characteristic of cells grown in control medium (data not shown). Thus, the G2/M block caused by genistein is reversible.

3.2. Effects of genistein on Cdc2 Tyr-15 phosphorylation, Cdc2 protein expression, and Cdc2-associated histone H1 kinase activity

Previous research has indicated that Cdc2 Tyr-15 phosphorylation, expression, and Cdc2- associated kinase activity are important components regulating the transition of cells from the G2 to the M phase [30]. Therefore, we determined whether genistein treatment influenced the Tyr-15 phosphorylation status of Cdc2, the expression of Cdc2, or Cdc2 activity. In MCF-10F cells, exposure to genistein for 4 and 8 hr resulted in a 2- to 3-fold increase in Cdc2 Tyr-15 phosphorylation (Fig. 3A, top panel) with no significant change in total expression levels (Fig. 3A, bottom panel), indicating that the change in Tyr-15 Cdc2 phosphorylation was not due to differences in loading or expression of Cdc2. However, treatment of MCF-10F cells with genistein for 24 hr markedly decreased the detectable Tyr-15 phosphorylated form of Cdc2 (Fig. 3A, top panel). This decrease in Tyr-15 phosphorylation after genistein treatment for 24 hr was presumably due to a total decrease in Cdc2 expression levels (Fig. 3A, bottom panel). In MCF-10F cells, exposure to genistein resulted in a decrease in Cdc2 activity (Fig. 3, B and C). Cdc2 activity decreased to 33% of controls within 8 hr of treatment and was maintained for 24 hr ($P < 0.05$). As a control, immunoprecipitation with a non-specific mouse IgG resulted in no detectable Cdc2 histone H1 kinase activity. Thus, the genistein-induced inhibition of MCF-10F cell proliferation and G2/M cell cycle block is associated with an initial increase in Tyr-15 phosphorylation of Cdc2, followed by a decrease in the level of Cdc2 protein and a decrease in Cdc2 kinase activity. We observed no change in the expression level of Cdc2 after 8 hr of genistein treatment. Others have reported that genistein causes a decrease in Cdc2 activity and no change in expression of Cdc2 [19]. However, we observed that the genistein-induced inhibition of Cdc2 kinase activity, after 24 hr, is associated with a decrease in the total expression level of Cdc2.

3.3. Genistein up-regulation of p21$^{waf/cip1}$ and p53 expression in MCF-10F human mammary epithelial cells

To determine the mechanism for the inhibition of Cdc2 activity by genistein in MCF-10F cells, we examined which upstream regulators of Cdc2 might be affected by genistein. The cdk inhibitor p21$^{waf/cip1}$ is a universal cdk inhibitor, which functions in both the G1 and G2 phases of the cell cycle [34]. The kinetics for the effect of genistein on total cellular levels of p21$^{waf/cip1}$ in MCF-10F cells were examined by western blot analysis, as shown in Fig. 4, A and B. p21$^{waf/cip1}$ increased up to 5-fold above control levels after 4 hr of genistein treatment ($P < 0.05$). At 12 hr after genistein addition, a maximum level of p21$^{waf/cip1}$ expression (15-fold above control levels) was attained ($P < 0.05$). The expression levels of p21$^{waf/cip1}$ remained elevated above those of control cells 36 hr after genistein addition.

Previous reports have indicated that the up-regulation of p21$^{waf/cip1}$ by genistein may occur through a p53-independent mechanism [16]. To determine if genistein changed the

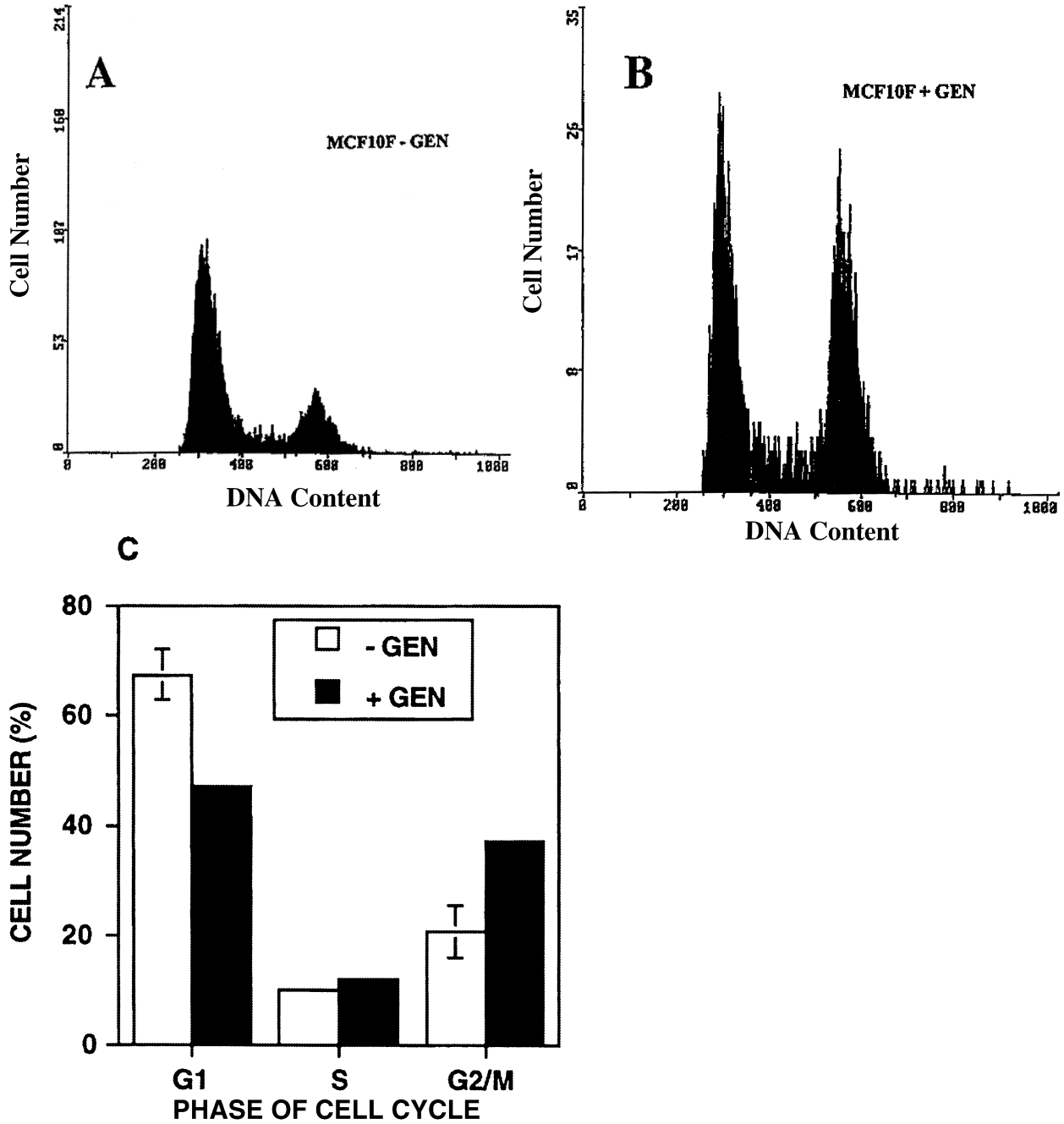

Fig. 2. Flow cytometric analyses of proliferating cultures of MCF-10F cells (~10^6) in the absence (A) or presence (B) of 30 μM genistein for 48 hr. The summary of cell cycle profiles in MCF-10F cells in the absence or presence of genistein is presented in panel C. The results shown in panels A and B are summarized by bar graphs (bars, means; error bars, coefficient of variation). Results are representative of two experiments. Where no error bars are shown, the differences in the means were too small for visualization.

expression level of p53, we stripped the blots shown in Fig. 4A and reprobed them with antisera raised against p53. As depicted in Fig. 4C, treatment of MCF-10F cells with genistein for 12 hr resulted in a 2.8-fold increase ($P < 0.05$) in p53 expression. This effect was not seen after 24 or 36 hr of genistein treatment. These results indicate that the up-regulation of $p21^{waf/cip1}$ may occur through a p53-dependent mechanism.

3.4. Genistein-mediated increase in Cdc2-associated $p21^{waf/cip1}$

To determine if the genistein-mediated decrease in Cdc2 kinase activity was associated with an increase in Cdc2-associated $p21^{waf/cip1}$, we treated cells with genistein or DMSO vehicle for different times. Cells were lysed and Cdc2 was immunoprecipitated. Immunoprecipitates were col-

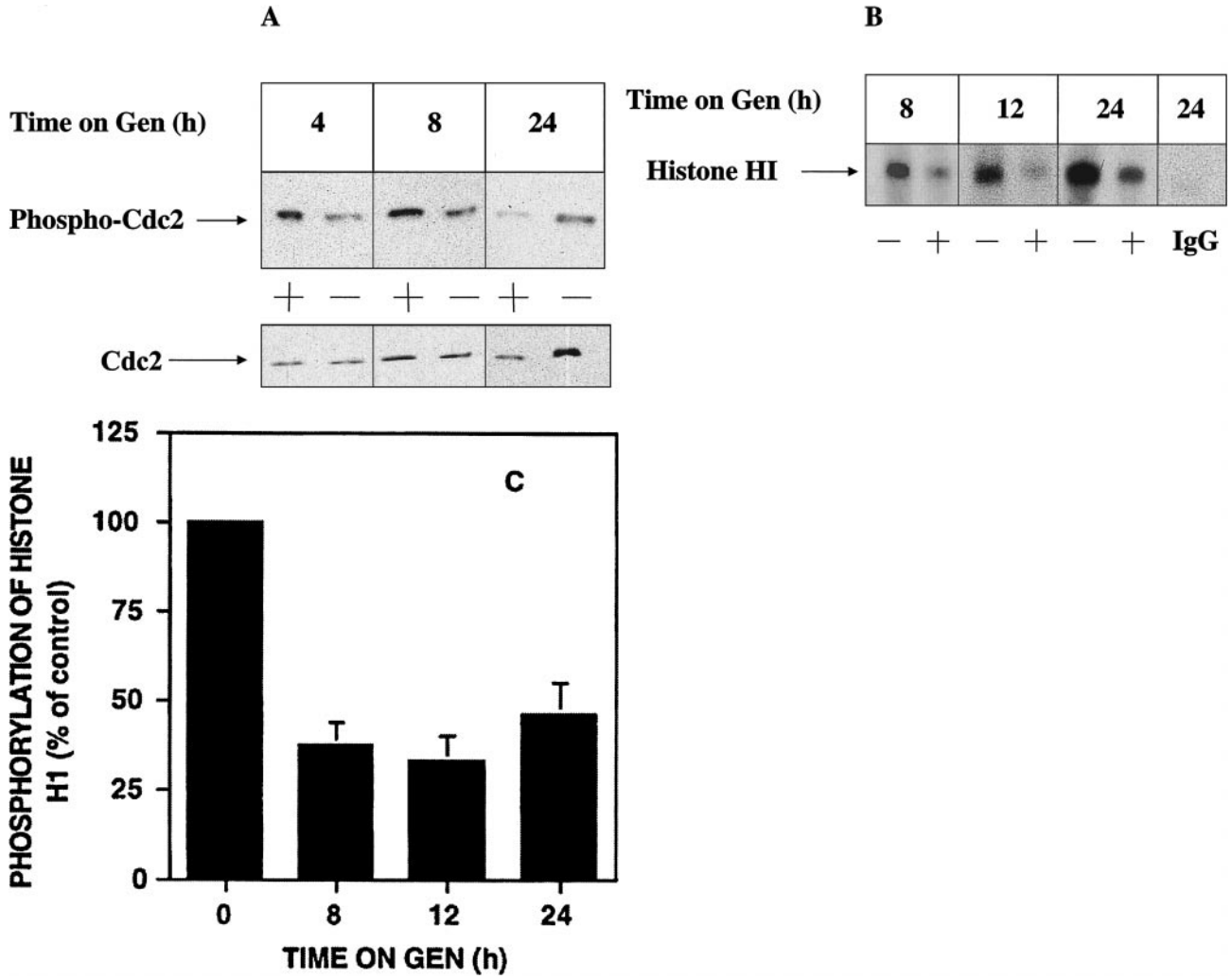

Fig. 3. Effect of genistein on $p34^{cdc2}$ Tyr-15 phosphorylation (A) and $p34^{cdc2}$ histone H1 kinase activity (B, C) in MCF-10F cells. (A) Kinetics for $p34^{cdc2}$ Tyr-15 phosphorylation, as determined by phospho-specific immunoblotting. Key: (+) in the presence of genistein; (−) in the absence of genistein (DMSO solvent control). (B) Kinetics for *in vitro* phosphorylation of histone H1 by $p34^{cdc2}$ in MCF-10F cells treated with genistein (45 μM) for the indicated times. No detectable Cdc2 kinase activity was present when all lysates were incubated with nonspecific mouse IgG. Key: (+) in the presence of genistein; (−) in the absence of genistein (DMSO solvent control). (C) Plot of densitometric scan results for histone H1 autoradiograph in panel B. Values are means ± SD (N = 3).

lected, and western blot analysis was performed for $p21^{waf/cip1}$. The data in Fig. 4D indicate that $p21^{waf/cip1}$ association with Cdc2 increased after 2 hr of genistein addition and was sustained for up to 4 hr after genistein treatment. Thus, the up-regulation of $p21^{waf/cip1}$ expression by genistein paralleled the genistein- mediated increase in Cdc2 association with $p21^{waf/cip1}$. Further, the kinetics for these effects may account, at least partially, for the decrease in Cdc2 activity. Results from concentration-dependent studies indicated that genistein induced the expression of $p21^{waf/cip1}$ in a concentration-dependent manner, with an EC_{50} value of 29 μM (Fig. 5).

3.5. Effect of genistein on $p27^{kip1}$ expression in MCF-10F human mammary epithelial cells

Another cdk inhibitor, $p27^{kip1}$, primarily functions in the G1 phase of the cell cycle by binding to cdk2–cyclin E/A complexes to inhibit cdk2 kinase activity [39,40]. Thus, it was of interest to examine the effects of genistein on total cellular levels of $p27^{kip1}$ in MCF-10F cells as well. We stripped the same immunoblot that was probed with the $p21^{waf/cip1}$ antibody, and probed it with an anti-$p27^{kip1}$ antibody. As shown in Fig. 6, after 12–36 hr of genistein treatment $p27^{kip1}$ levels were not increased significantly ($P > 0.05$), compared with the induction of $p21^{waf/cip1}$. Thus, the genistein-mediated inhibition of Cdc2 activity appears to be related more to the ability of genistein to increase $p21^{waf/cip1}$ than $p27^{kip1}$ levels.

3.6. Genistein down-regulation of Cdc25C protein expression in MCF-10F human mammary epithelial cells

In eukaryotic cells, entry into mitosis is catalyzed by the Cdc25C-dependent dephosphorylation of Tyr-15 in the

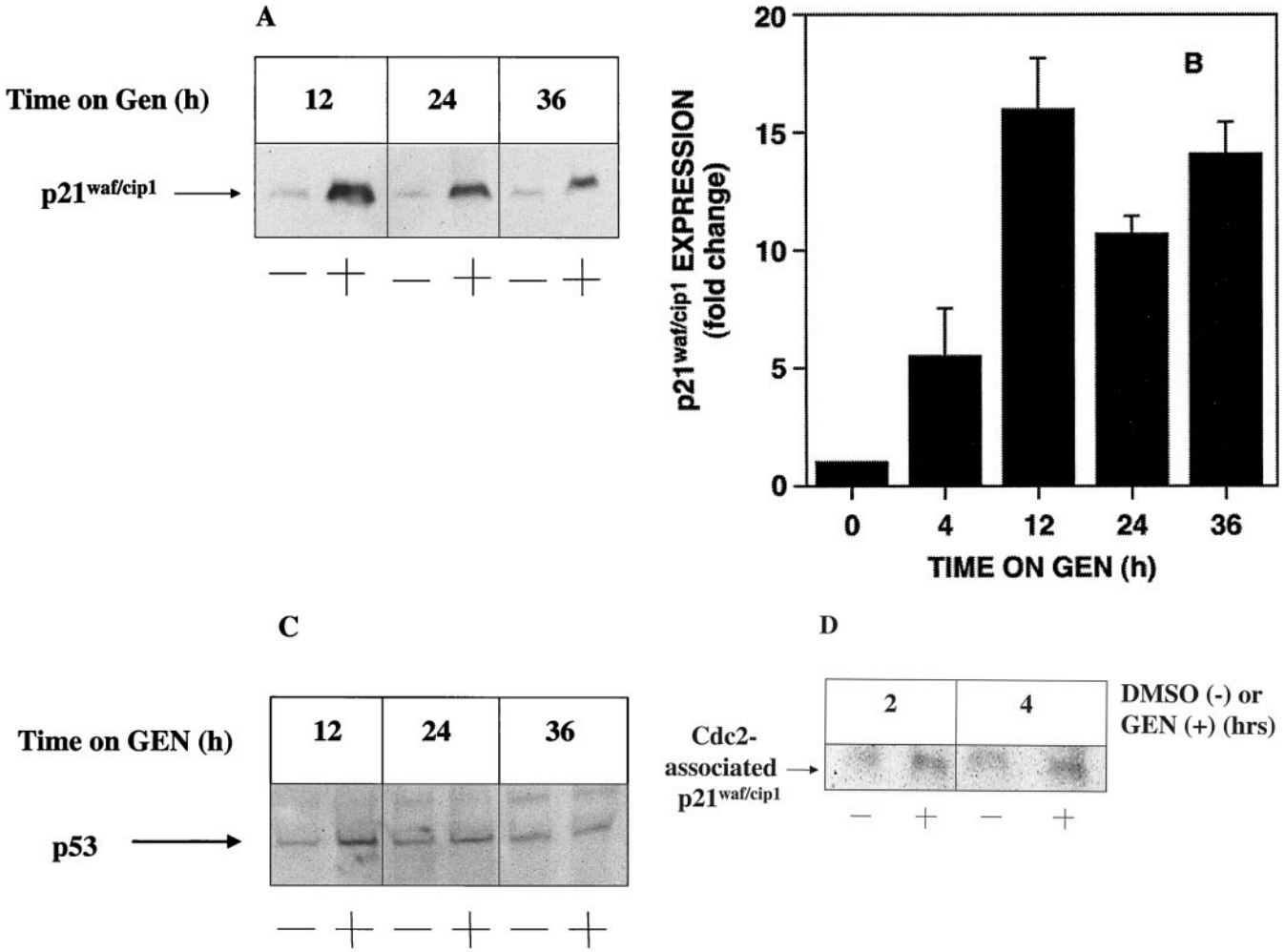

Fig. 4. Kinetics for the effect of genistein on $p21^{waf/cip1}$ expression (A, B), p53 expression (C), and $p34^{cdc2}$-associated $p21^{waf/cip1}$ (D) in MCF-10F cells. (A) Autoradiograph of $p21^{waf/cip1}$ expression in MCF-10F cells treated with (+) or without (−) genistein (DMSO solvent control) for the indicated times. (B) Plot of densitometric scan results for $p21^{waf/cip1}$ autoradiograph in panel A. Values are means ± SD (N = 3). (C) Western blot analysis of p53 expression in MCF-10F cells treated with (+) or without (−) genistein (DMSO solvent control) for various times. (D) Total cell lysates were subjected to immunoprecipitation with anti-$p34^{cdc2}$ followed by SDS–PAGE and immunoblot analysis with anti-$p21^{waf/cip1.}$ Key: (+) in the presence of genistein; (−) in the absence of genistein (DMSO solvent control).

Cdc2 subunit of the cyclin B/Cdc2 complex, which activates its protein kinase activity [30]. Thus, it was of interest to examine the effects of genistein on total cellular levels of the phosphatase Cdc25C in MCF-10F cells. As shown in Fig. 7, genistein treatment of MCF-10F cells resulted in a time-dependent decrease in Cdc25C levels. Cdc25C expression decreased to 25% of controls by 12 hr after genistein addition, and was inhibited by 80% within 24–36 hr of treatment. Results from concentration-dependent studies indicated that genistein down-regulated the expression of Cdc25C in a concentration-dependent manner, with an IC_{50} value of 17.5 μM (Fig. 8). Thus, these results suggest that decreased Cdc25C expression may be involved in the genistein-induced reduction in Cdc2 kinase activity in MCF-10F cells.

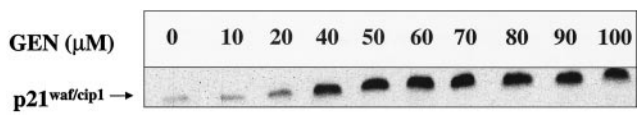

Fig. 5. Concentration-dependent effects of genistein on the $p21^{waf/cip1}$ expression in MCF-10F cells. Cells were incubated with various concentrations of genistein for 24 hr. This experiment was performed at least twice.

4. Discussion

We have demonstrated in the present study that exposure of nonneoplastic human mammary epithelial cells to genistein results in a concentration-dependent inhibition of proliferation and an accumulation of cells at G2/M. Others [16] have observed a G2 block in the human breast cancer cell cycle due to genistein, but at much higher concentra-

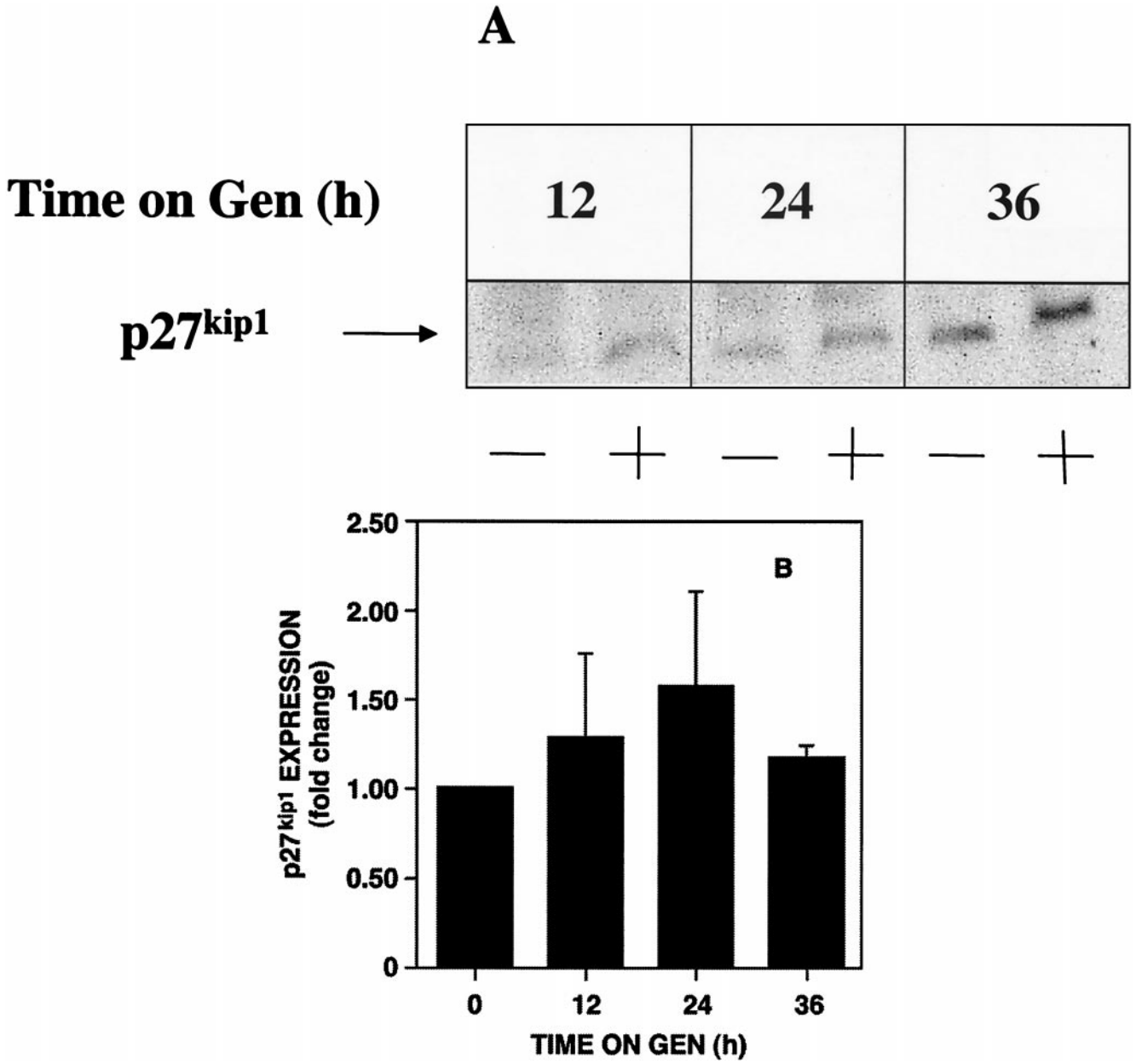

Fig. 6. Kinetics for the effect of genistein on $p27^{kip1}$ expression in MCF-10F cells. (A) Autoradiograph of $p27^{kip1}$ expression in MCF-10F cells treated with (+) or without (−) genistein (DMSO solvent control) for the indicated times. (B) Plot of densitometric scan results for $p27^{kip1}$ autoradiograph in panel A. Values are means ± SD (N = 3).

tions (74 μM) than those examined in the present studies. Our results are similar to those of McIntyre and Sylvester [41] who, using primary human mammary epithelial cells, reported that genistein inhibits cell proliferation by 50% at a concentration of ~25 μM. Comparing the effects of genistein on normal mammary epithelial cells to those on MCF-7 breast cancer cells, Peterson *et al.* [25] reported that normal mammary epithelial cells are more responsive to growth inhibition by genistein than are MCF-7 breast cancer cells. This was presumably due to a change in the metabolism of genistein. Thus, genistein may have less antiproliferative efficacy toward human breast cancer cells than nonneoplastic human breast cells. The G2 block induced by genistein in MCF-10F cells and the magnitude of cell growth inhibition that we observed in this nonneoplastic breast cell line are similar to the action of genistein reported by others for human breast and prostate cancer cell lines [5,15–17,42]. Collectively, this suggests that genistein is not a selective inhibitor of neoplastic human breast cells compared with those that are nonneoplastic.

The observation that genistein increases the expression of the tumor suppressor p53 in MCF-10F cells is consistent with inhibition by genistein of cell proliferation. Previous reports have indicated that the up-regulation of $p21^{waf/cip1}$ can occur through a p53-independent pathway [16]. However, our p53 results are in contrast to other reports indicat-

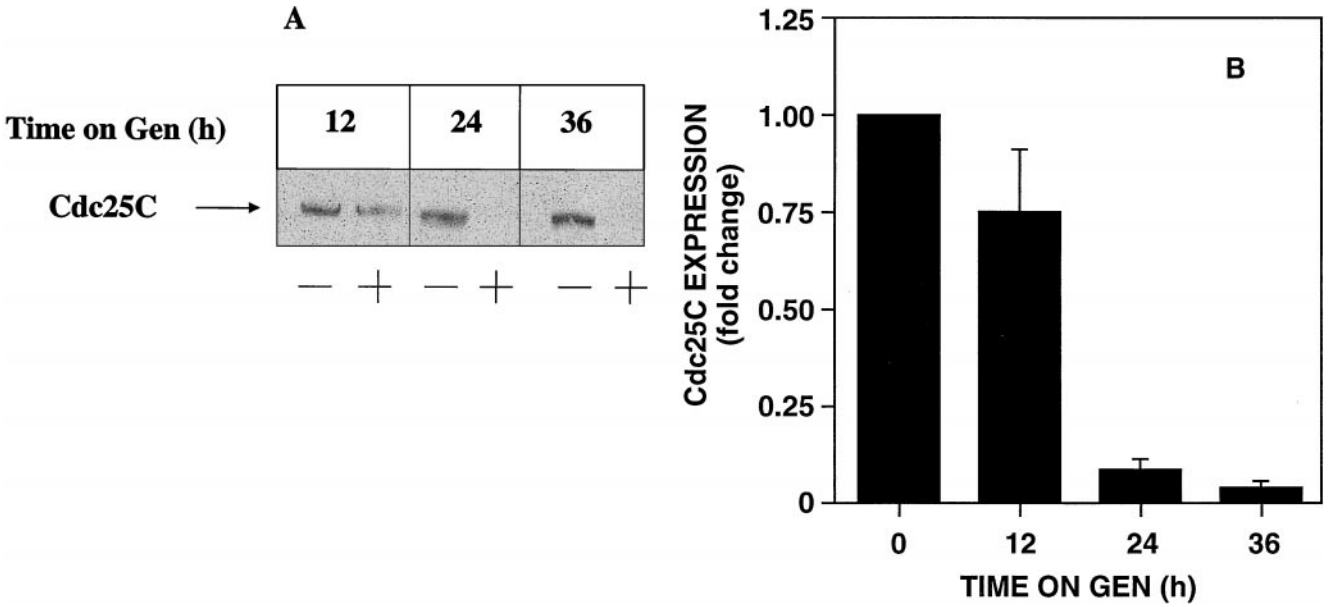

Fig. 7. Kinetics for the effect of genistein on Cdc25C protein expression in MCF-10F cells. (A) Autoradiograph of Cdc25C protein expression in MCF-10F cells treated with (+) or without (−) genistein (DMSO solvent control) for the indicated times. (B) Plot of densitometric scan results for Cdc25C autoradiograph in panel A. Values are means ± SD (N = 3).

ing that genistein does not induce $p21^{waf/cip1}$ through p53 [43–45]. These conflicting results may be due to differences in transformation status or cell-type mutations in p53.

The increase in the number of cells present in the G2 phase of the cell cycle was preceded by genistein-mediated up-regulation of $p21^{waf/cip1}$ expression and reduced Cdc2 kinase activity. This is consistent with the fact that in eukaryotic cells movement through the G2/M transition is negatively regulated by Cdc2 activity [46]. The results of the present studies indicate that this decrease in Cdc2 kinase activity may largely be explained by a sizable increase in the expression of the cdk inhibitor $p21^{waf/cip1}$, as well as in enhanced association of $p21^{waf/cip1}$ with Cdc2. Genistein has also been reported to increase $p21^{waf/cip1}$ expression in MDA-MB-231 and MCF-7 breast cancer cells [16]. In addition, Choi *et al.* [47] have demonstrated an enhanced association between $p21^{waf/cip1}$ and Cdc2 in genistein-treated MDA-MB-231 and MCF-7 breast cancer cells. Furthermore, other growth inhibitors have been shown to increase the association of other cdks with $p21^{waf/cip1}$ [34,48]. Additionally, the observed genistein-induced decrease in Cdc2 activity in MCF-10F cells in our studies may be due, in part, to the action of Cdc25C phosphatase, the total protein levels of which decreased substantially after genistein treatment. It has been reported that the phosphorylation status of Cdc2 is determined in large measure by the relative activities of Cdc25C phosphatase and Wee1 kinase [39]. It is possible that exposure of these MCF-10F cells to genistein is altering the balance of phosphatase and kinase activities of the above enzymes. Of additional interest is the temporal effect of genistein treatment on phospho-Cdc2 levels and total expression of Cdc2. Initially, Cdc2 kinase inhibition due to genistein exposure may be partly explained by increased Cdc2 Tyr-15 phosphorylation and $p21^{waf/cip1}$ up-regulation. However, subsequent inhibition of kinase activity appears largely associated with a decrease in total Cdc2 protein. Down-regulation of Cdc2 expression has been reported to parallel decreased Cdc2 histone H1 kinase activity in rat alveolar epithelial cells [49]. However, the effect of genistein on down-regulation of Cdc2 protein expression has not been reported for transformed or nontransformed breast epithelial cells [47,50] and merits further investigation.

The data generated from the present experiments

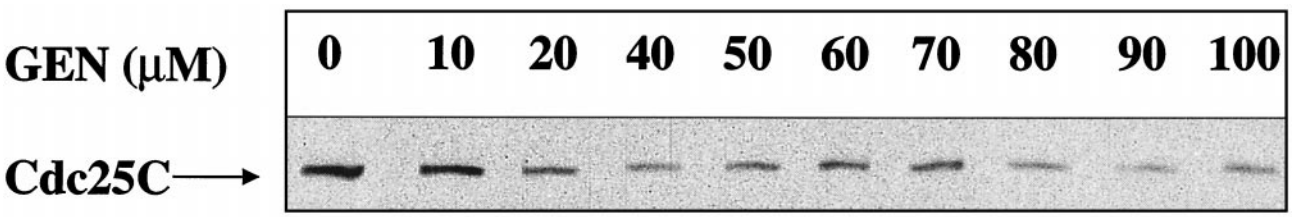

Fig. 8. Concentration-dependent effects of genistein on Cdc25C expression in MCF-10F cells. Cells were incubated with various concentrations of genistein for 24 hr. This experiment was performed at least twice.

showing regulation of cell cycle progression, increased $p21^{waf/cip1}$ and p53 expression, and no detectable morphological changes associated with apoptosis by genistein in MCF-10F cells demonstrate the need for future experiments designed to understand the role of p53, $p21^{waf/cip1}$, and growth regulation by phytochemicals and other chemopreventive agents [51–54].

Data from the present studies add to an increasing body of evidence that genistein can alter specific cell-signaling events in a variety of cell lines. For example, genistein-induced G2/M arrest of ER-positive and ER-negative human breast carcinoma cell lines was associated with increased $p21^{waf/cip1}$ expression due largely to greater mRNA stability [16]. It also has been observed that genistein can inhibit Cdc2 kinase activity in MDA-MB-468 human breast cancer cells [50], Shc tyrosine phosphorylation and MAP kinase activity in SKBR3 and MCF-7 breast cancer cell lines [12], and 12-*O*-tetradecanoylphorbol-13-acetate-induced mitogen-activated protein (MAP) kinase and $p70^{S6}$ kinase activities in CV-1 cells [55]. Our report suggests that nonneoplastic human breast cells also are sensitive to genistein-induced growth inhibition, which is associated, in part, with changes in Cdc2 Tyr-15 phosphorylation, Cdc2 kinase activity and expression, and the expression of $p21^{waf/cip1}$ and Cdc25C.

Our findings of cell cycle arrest of nonneoplastic MCF-10F cells by genistein should be considered in strategies of breast cancer prevention, since genistein may be nonselective in causing growth inhibition in both neoplastic and nonneoplastic human breast cells. Of additional interest is that, although nonneoplastic, the MCF-10F cells are immortalized, which has been suggested to be an early event in the neoplastic transformation of human mammary epithelial cells [56–58]. Thus, genistein also may prove useful as an inhibitor of cells undergoing early events in breast cell transformation and progressive human proliferative breast disease. This possible use of genistein warrants further investigation.

References

[1] Mazur WM, Duke JH, Wähälä K, Rashu S, Adlercreutz H. Isoflavonoids and lignans in legumes: nutritional and health aspects in humans. J Nutr Biochem 1998;9:193–200.

[2] Stoll BA. Eating to beat breast cancer: potential role for soy supplements. Ann Oncol 1997;8:223–5.

[3] Wei H, Bowen R, Cai Q, Barnes S, Wang Y. Antioxidant and antipromotional effects of the soybean isoflavone genistein. Proc Soc Exp Biol Med 1995;208:124–30.

[4] Barnes S, Peterson TG. Biochemical targets of the isoflavone genistein in tumor cell lines. Proc Soc Exp Biol Med 1995;208: 103–8.

[5] Barnes S. Effect of genistein on *in vitro* and *in vivo* models of cancer. J Nutr 1995;125(Suppl 3):777S–83S.

[6] Constantinou AI, Mehta RG, Vaughan A. Inhibition of *N*-methyl-*N*-nitrosourea-induced mammary tumors in rats by the soybean isoflavones. Anticancer Res 1996;16:3293–8.

[7] Hawrylewicz EJ, Zapata JJ, Blair WH. Soy and experimental cancer: animal studies. J Nutr 1995;125(Suppl 3):698S–708S.

[8] Peterson G. Evaluation of the biochemical targets of genistein in tumor cells. J Nutr 1995;125(Suppl 3):784S–9S.

[9] Wang TT, Sathyamoorthy N, Phang JM. Molecular effects of genistein on estrogen receptor mediated pathways. Carcinogenesis 1996;17:271–5.

[10] Mäkelä S, Davis VL, Tally WC, Korkman J, Salo L, Vihko R, Santti R, Korach KS. Dietary estrogens act through estrogen receptor-mediated processes and show no antiestrogenicity in cultured breast cancer cells. Environ Health Perspect 1994;102:572–8.

[11] Constantinou A, Kiguchi K, Huberman E. Induction of differentiation and DNA strand breakage in human HL-60 and K-562 leukemia cells by genistein. Cancer Res 1990;50:2618–24.

[12] Clark JW, Santos-Moore A, Stevenson LE, Frackelton AR. Effects of tyrosine kinase inhibitors on the proliferation of human breast cancer cell lines and proteins important in the ras signaling pathway. Int J Cancer 1996;65:186–91.

[13] Brown A, Jolly P, Wei H. Genistein modulates neuroblastoma cell proliferation and differentiation through induction of apoptosis and regulation of tyrosine kinase activity and N-*myc* expression. Carcinogenesis 1998;19:991–7.

[14] Wang Y, Yaping E, Zhang X, Lebwohl M, DeLeo V, Wei H. Inhibition of ultraviolet B (UVB)-induced c-*fos* and c-*jun* expression *in vivo* by a tyrosine kinase inhibitor genistein. Carcinogenesis 1998; 19:649–54.

[15] Matsukawa Y, Marui N, Sakai T, Satomi Y, Yoshida M, Matsumoto K, Nishino H, Aoike A. Genistein arrests cell cycle progression in G_2-M. Cancer Res 1993;53:1328–31.

[16] Shao AM, Alpaugh ML, Fontana JA, Barsky SH. Genistein inhibits proliferation similarly in estrogen receptor-positive and negative human breast carcinoma cell lines characterized by $P21^{waf/cip1}$ induction, G2/M arrest, and apoptosis. J Cell Biochem 1998;69:44–54.

[17] Traganos F, Ardelt B, Halko N, Bruno S, Darzynkiewicz Z. Effects of genistein on the growth and cell cycle progression of normal human lymphocytes and human leukemic MOLT-4 and HL-60 cells. Cancer Res 1992;52:6200–8.

[18] Lepley DM, Li B, Birt DF, Pelling JC. The chemopreventive flavonoid apigenin induces G_2/M arrest in keratinocytes. Carcinogenesis 1996;17:2367–75.

[19] Lepley DM, Pelling JC. Induction of p21/WAF1 and G_1 cell-cycle arrest by the chemopreventive agent apigenin. Mol Carcinog 1997; 19:74–82.

[20] Kaur G, Stetler-Stevenson M, Sebers S, Worland P, Sedlacek H, Myers C, Czech J, Naik R, Sausville E. Growth inhibition with reversible cell cycle arrest of carcinoma cells by the flavone L86–8275. J Natl Cancer Inst 1992;84:1736–40.

[21] Monti E, Sinha B. Antiproliferative effect of genistein and adriamycin against estrogen-dependent and -independent human breast carcinoma cell lines. Anticancer Res 1994;14:1221–6.

[22] Krygier A, Murley J, Mehta R, Constantinou A. Induction of human adenocarcinoma cell differentiation by the phytoestrogen genistein is independent of its antiestrogenic function. Int J Oncol 1997;10:753–7.

[23] Peterson G, Barnes S. Genistein inhibits both estrogen and growth factor-stimulated proliferation of human breast cancer cells. Cell Growth Differ 1996;7:1345–51.

[24] Santell RC, Chang YC, Nair MG, Helferich WG. Dietary genistein exerts estrogenic effects upon the uterus, mammary gland and the hypothalamic/pituitary axis in rats. J Nutr 1997;127:263–9.

[25] Peterson T, Coward L, Kirk M, Falany C, Barnes S. The role of metabolism in mammary epithelial cell growth inhibition by the isoflavones genistein and biochanin A. Carcinogenesis 1996;17:1861–9.

[26] Soule H, Maloney T, Wolman S, Peterson W, Brenz R, McGrath C, Russo J, Pauley R, Jones R, Brooks S. Isolation and characterization

of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 1990;50:6075–86.
[27] King R, Jackson P, Kirschner M. Mitosis in transition. Cell 1994;79: 563–71.
[28] Solomon MJ, Glotzer M, Lee TH, Phillippe M, Kirscher MW. Cyclin activation of $p34^{cdc2}$. Cell 1990;63:1013–24.
[29] McGowan CH, Russell P. Human Wee1 kinase inhibits cell division by phosphorylating $p34^{cdc2}$ exclusively on Tyr15. EMBO J 1993;12: 75–85.
[30] Gautier J, Solomon M, Booher R, Bazan J, Kirschner M. Cdc25 is a specific tyrosine phosphatase that directly activates Cdc2. Cell 1991; 65:197–211.
[31] Alhasan SA, Ensley JF, Sarkar FH. Genistein induced molecular changes in a squamous cell carcinoma of the head and neck cell line. Int J Oncol 2000;16:333–8.
[32] Pines J, Hunter T. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. J Cell Biol 1991;115:1–17.
[33] Lallemand F, Courilleau D, Buquet-Fagot C, Atfi A, Montagne MN, Mester J. Sodium butyrate induces G2 arrest in the human breast cancer cells MDA-MB-231 and renders them competent for DNA rereplication. Exp Cell Res 1999;247:432–40.
[34] Barboule N, Lafon C, Chadebech P, Vidal S, Valette A. Involvement of p21 in the PKC-induced regulation of the G2/M cell cycle transition. FEBS Lett 1999;444:32–7.
[35] Sobottka S, Berger M. Assessment of antineoplastic agents by MTT assay: partial underestimation of antiproliferative properties. Cancer Chemother Pharmacol 1992;30:385–93.
[36] Frey RS, Mulder KM. Involvement of extracellular signal-regulated kinase 2 and stress-activated protein kinase/Jun *N*-terminal kinase activation by transforming growth factor β in the negative growth control of breast cancer cells. Cancer Res 1997;57:628–33.
[37] Hartsough MT, Frey RS, Zipfel PA, Buard A, Cook SJ, McCormick F, Mulder KM. Altered transforming growth factor β signaling in epithelial cells when ras activation is blocked. J Biol Chem 1996; 271:22368–75.
[38] Slingerland JM, Hengst L, Pan CH, Alexander D, Stampfer MR, Reed SI. A novel inhibitor of cyclin-Cdk activity detected in transforming growth factor β-arrested epithelial cells. Mol Cell Biol 1994;14: 3683–94.
[39] Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A. $p27^{Kip1}$, a cyclin-Cdk inhibitor, links transforming growth factor-β and contact inhibition to cell cycle arrest. Genes Dev 1994; 8:9–22.
[40] Toyoshima H, Hunter T. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell 1994;78:67–74.
[41] McIntyre BS, Sylvester PW. Genistein and erbstatin inhibition of normal mammary epithelial cell proliferation is associated with EGF-receptor down-regulation. Cell Prolif 1998;31:35–46.
[42] Pagliacci M, Smacchia M, Migliorati G, Grignani F, Riccardi C, Nicoletti I. Growth-inhibitory effects of the natural phytoestrogen genistein in MCF-7 human breast cancer cells. Eur J Cancer 1994; 30A:1675–82.
[43] Lian F, Li Y, Bhuiyan M, Sarker FH. p53-independent apoptosis induced by genistein in lung cancer cells. Nutr Cancer 1999;33:125–31.
[44] Li Y, Upadhyay S, Bhuiyan M, Sarkar FH. Induction of apoptosis in breast cancer cells MDA-MD-231 by genistein. Oncogene 1999;18: 3166–72.
[45] Choi YH, Lee WH, Park KY, Zhang L. p53-independent induction of p21 (WAF1/CIP1), reduction of cyclin B1 and G2/M arrest by the isoflavone genistein in human prostate carcinoma cells. Jpn J Cancer Res 2000;91:164–73.
[46] Norbury C, Nurse P. Animal cell cycles and their control. Annu Rev Biochem 1992;61:441–70.
[47] Choi YH, Zhang L, Lee WH, Park KY. Genistein-induced G2/M arrest is associated with the inhibition of cyclin B1 and the induction of p21 in human breast carcinoma cells. Int J Oncol 1998;13:391–6.
[48] Yue J, Buard A, Mulder KM. Blockade of TGFβ3 up-regulation of $p27^{Kip1}$ and $p21^{Cip1}$ by expression of RasN17 in epithelial cells. Oncogene 1998;17:47–55.
[49] Wu F, Buckley S, Bui KC, Warburton D. Differential expression of cyclin D2 and cdc2 genes in proliferating and nonproliferating alveolar epithelial cells. Am J Respir Cell Mol Biol 1995;12:95–103.
[50] Balabhadrapathruni S, Thomas TJ, Yurkow EJ, Amenta PS, Thomas T. Effects of genistein and structurally related phytoestrogens on cell cycle kinetics and apoptosis in MDA-MB-468 human breast cancer cells. Oncol Rep 2000;7:3–12.
[51] Telang NT, Katadare M, Bradlow HL, Osborne M, Fishman J. Inhibition of proliferation and modulation of estradiol metabolism: novel mechanisms for breast cancer prevention by the phytochemical indole-3-carbinol. Proc Soc Exp Med 1997;216:245–52.
[52] Jinno H, Steiner MG, Mehta RG, Osborne MP, Teland NT. Inhibition of aberrant proliferation and induction of apoptosis in HER-2/*neu* oncogene transformed human mammary epithelial cells by *N*-(4-hydroxyphenyl)retinamide. Carcinogenesis 1999;20:229–36.
[53] Katdare M, Osborne MP, Telang NT. Inhibition of aberrant proliferation and induction of apoptosis in pre-neoplastic human mammary epithelial cells by natural phytochemicals. Oncol Rep 1998;5:311–5.
[54] Katdare M, Jinno H, Osborne M, Telang NT. Negative growth regulation of oncogene-transformed human breast epithelial cells by phytochemicals. Ann NY Acad Sci 1999;889:247–52.
[55] Losiewicz M, Carlson B, Kaur G, Sausville E, Worland P. Potent inhibition of cdc2 kinase activity by the flavonoid L86–8275. Biochem Biophys Res Commun 1994;201:589–95.
[56] Tseng C, Verma A. Inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-induced ornithine decarboxylase activity by genistein, a tyrosine kinase inhibitor. Mol Pharmacol 1996;50:249–57.
[57] Miller F, Soule H, Tait L, Pauley R, Wolman S, Dawson P, Heppner G. Xenograft model of progressive human proliferative breast disease. J Natl Cancer Inst 1993;85:1725–32.
[58] Stampfer M, Bodnar A, Garbe J, Wong M, Pan A, Villeponteau B, Yaswen P. Gradual phenotypic conversion associated with immortalization of cultured human mammary epithelial cells. Mol Biol 1997; 8:2391–405.