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Genistein arrests hepatoma cells at G2/M phase: involvement of ATM activation and upregulation of p21$^{waf1/cip1}$ and Wee1

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Abstract

Genistein, a soy isoflavone, has a wide range of biological actions that suggest it may be of use in cancer prevention. We have recently reported that it arrests hepatoma cells at G2/M phase and inhibits Cdc2 kinase activity. In the present study, we examined the signaling pathway by which genistein modulates Cdc2 kinase activity in HepG2 cells and leads to G2/M arrest, and found that it caused an increase in both Cdc2 phosphorylation and expression of the Cdc2-active kinase, Wee1. Genistein also enhanced the expression of the cell cycle inhibitor, p21$^{waf1/cip1}$, which interacts with Cdc2. Furthermore, phosphorylation/inactivation of Cdc25C phosphatase, which dephosphorylates/activates Cdc2, was increased. Genistein enhanced the activity of the checkpoint kinase, Chk2, which phosphorylates/inactivates Cdc25C, induced accumulation of p53, and activated the ataxia-telangiectasia-mutated (ATM) gene. Caffeine, an ATM kinase inhibitor, inhibited these effects of genistein on Chk2, p53, and p21$^{waf1/cip1}$. These findings suggest that the effect of genistein on G2/M arrest in HepG2 cells is partly due to ATM-dependent Chk2 activation, an increase in Cdc2 phosphorylation/inactivation as a result of induction of Wee1 expression, and a decrease in Cdc2 activity as a result of induction of p21$^{waf1/cip1}$ expression.

Keywords: Genistein; HepG2; ATM; p53; p21$^{waf1/cip1}$; Wee1

1. Introduction

The phytoestrogen isoflavones present in soybeans have a wide range of properties that may contribute to their cancer chemoprotective actions [1]; these include an inhibitory effect on tyrosine kinases, DNA topoisomerases I and II, and ribosomal S6 kinase, anti-estrogenicity, antioxidant activity, anti-angiogenesis activity, suppression of cell proliferation, induction of differentiation, and modulation of apoptosis [2–5]. In addition, genistein (5,7,4′-trihydroxyisoflavone), one of the most active of these compounds, is an effective chemopreventive agent in dimethylbenz[α]anthracene-induced mammary carcinogenesis in neonatal rats [6]. Our previous study [7] showed that genistein has an antiproliferative effect on human hepatoma cancer cell lines, as previously reported for a number of other cell types [8,9]. However, its mechanism of action and its molecular targets on human hepatoma cells remain unclear.

Cell cycle checkpoints are biochemical pathways that ensure the orderly and timely progression and completion of critical events, such as DNA replication and chromosome segregation. The ultimate target of the G2 checkpoint signaling pathway is the cyclin-dependent kinase (Cdk) complex, Cdk1-cyclin B1. Cdc2, a Cdk1 first discovered in *Schizosaccharomyces pombe*, forms a heterodimeric complex with cyclin B1 which is maintained in an inactive form by phosphorylation of residues Thr-14 and Tyr-15 in the ATP-binding domain of Cdc2 by Wee1 kinase [10,11] and is converted to an active form by dephosphorylation of these residues by the dual specificity phosphatase, Cdc25C [12]. This dephosphorylation/activation is an absolute

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Abbreviations: DMEM, Dulbecco's modified Eagle medium; ATM, ataxia-telangiectasia-mutated; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence.

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requirement for the onset of mitosis. Recently, it has been shown that Cdc25C is negatively regulated by phosphorylation of its Ser-216 residue during interphase or in response to DNA damage or incomplete DNA replication [13]. Phosphorylation of this residue creates a binding site for 14-3-3 proteins, which are believed to be responsible for the nuclear export of Cdc25C and the subsequent inhibition of nuclear Cdk1 dephosphorylation. Two checkpoint kinases, Chk1 and Chk2, have been recently identified in humans and shown to phosphorylate Cdc25C on Ser-216 [13,14]. Chk1 is activated in γ-irradiated HeLa cells [14], whereas Chk2 is involved in both the replication and DNA damage checkpoints [15]. Although the initial study [14] demonstrated that Chk1 could be activated by ionizing radiation, more recent studies clearly showed that this activation is only observed at high doses and that Chk2 is markedly more responsive to ionizing radiation than Chk1 [15,16]. The response to DNA damage is dependent on the ATM protein signaling pathway [17]. ATM, a member of the phosphatidylinositol 3-kinase (PI 3-K) protein family [18], is required for the activation of p53, a tumor suppressor protein, and of Chk2, which results, respectively, in cell cycle arrest at G1/S or G2/M [19].

A 21-kD protein, $p21^{waf1/cip1}$, is a component of Cdk-cyclin complexes and can modulate the activity of a number of Cdks [20]. Association of $p21^{waf1/cip1}$ with Cdk-cyclin complexes results in decreased Cdk activity, which, in turn, inhibits the phosphorylation/inactivation of specific endogenous substrates, such as the retinoblastoma protein, which are required for progression through the cell cycle [21]. Additionally, $p21^{waf1/cip1}$ is a universal Cdk inhibitor and causes cell cycle arrest at G1/S or G2/M [20,22].

We have recently reported that genistein arrests hepatoma cells at G2/M phase and inhibits Cdc2 kinase activity [7]. The aims of the present study were, therefore, to examine the effectiveness of genistein in modulating Wee1 and $p21^{waf1/cip1}$ expression and Cdc25C phosphorylation status, and to address the question of whether genistein exerts these effects by activating an ATM kinase and, if so, whether this activation involves p53 or Chk2.

2. Materials and methods

2.1. Reagents

Genistein, DMSO, and protein A-Sepharose were purchased from Sigma Chemical Co. Horseradish peroxidase-conjugated secondary antibodies and antibodies against p53, $p21^{waf1/cip1}$, Chk2, Cdc25C, Cdc25C-P, Cdc2, Cdc2-P, cyclin B1, and β-actin were purchased from Santa Cruz Biotechnology Inc. Anti-ATM antibody and PHAS-1 were purchased from Oncogene Research Products. Histone H1 was purchased from Calbiochem-Novabiochem. Cell growth media and serum were obtained from GIBCO BRL. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from NEN Life Science Products and the chemiluminescence detection kit from PerkinElmer Life Sciences Inc.

2.2. Cell culture

The human hepatoma cell line, HepG2, obtained from the American Type Culture Collection, was grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were seeded at 1×10^4 cells/well and grown at 37° in a 5% CO_2 incubator before being used in the different experiments.

2.3. Cell cycle analysis

The distribution of cells at different stages in the cell cycle was estimated by flow cytometric DNA analysis as described previously [9]. Briefly, 1×10^6 cells were incubated overnight at 37° in 10 cm plastic dishes, then for 24 hr with or without various concentrations of isoflavones, detached using trypsin–EDTA, washed twice with cold PBS, pH 7.4 (PBS), and fixed at 4° with 70% ethanol/30% PBS. The fixed cells were incubated for 30 min at 4° in the dark with 1 mL of fluorochrome DNA staining solution containing 40 μg of propidium iodide and 0.1 mg of RNase A and analyzed on a FACS cytometer. The percentage of cells in each cell cycle phase (G0/G1, S, or G2/M) was calculated using Lysis II Software, a minimum of 1.5×10^4 cells/sample being evaluated.

2.4. Western blotting

Cytosolic extracts were prepared by suspending 1×10^7 cells in 1 mL of ice-cold lysis buffer [50 mM Tris–HCl, pH 7.4, 0.25 M NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 1 × cocktail of protease inhibitors (Sigma), 1 mM phenylmethylsulfonyl fluoride, and 1 mg/L aprotinin], and incubated on ice for 30 min. After centrifugation at 8000 *g* for 20 min at 4°, the protein in the supernatant was quantitated using a protein assay kit from Bio-Rad Laboratories and a sample (60 μg) was electrophoresed on 12% SDS–polyacrylamide gels, then transferred to nitrocellulose membranes. The membranes were blocked for 90 min at room temperature in PBS plus 0.5% Tween 20 containing 5% fat-free powdered milk, then incubated for 2 hr at room temperature with rabbit polyclonal antibodies against human Chk2, Cdc2, Cdc2-P, Cdc25C-P, p21, or β-actin or mouse monoclonal anti-human p53 antibody. After washing, the membranes were incubated for 90 min at 25° with the appropriate horseradish peroxidase-labeled secondary antibody, and bound antibody visualized and quantified by chemiluminescence detection. β-Actin was used as the internal control. The amount of the protein of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units of β-actin, then the density of the band was expressed as the relative density compared to that in untreated cells (control), taken as 100%.

2.5. cDNA microarray analysis

HepG2 cells were cultured for 12 hr in DMEM containing 20 μg/mL genistein, then total RNA was extracted using REzol™ reagent and treated with DNase I. Fifty micrograms of DNase I-treated total RNA was reverse-transcribed to generate cDNA probes labeled with the fluorescent dye Cy3 (control sample) or Cy5 (genistein-treated sample). The labeled probes were hybridized to a commercial cDNA microarray containing ~8000 immobilized fragments of cancer and immunity related genes (Asia BioInnovations Corporation) [23]. The fluorescence intensities of the Cy3- and Cy5-labeled probes bound to each target were measured and scanned separately using a Genepix 4000B Array Scanner (Axon Instruments). Data analysis was performed using the supplied GenePix Pro 3.0.5.56 software (Axon Instruments). The results were normalized for the labeling and detection efficiencies of the two fluorescence dyes (Cy3 is green and Cy5 red), then used to detect differential gene expression in the control and treated cell samples.

2.6. ATM and Cdc2 kinase assay

ATM kinase [24] and Cdc2 kinase [9] assays were performed as described previously. Briefly, extracts from 1×10^7 cells were prepared by addition of 500 μL of lysis buffer for 10 min, followed by centrifugation for 15 min at 1500 *g* at 4°. The protein content of the supernatant was estimated by the Bio-Rad method and samples (100 μg) were incubated for 3 hr at 4° with 2 μg of monoclonal anti-ATM or anti-Cdc2 kinase antibody in a volume of 200 μL, then 200 μL of protein A-Sepharose was added and the sample mixed for 2 hr at 4° on a rotator.

The ATM kinase assay was performed for 30 min at 30° on the protein A-Sepharose-bound immune complexes resuspended in 500 μL of 10 mM HEPES, pH 7.5, 50 mM β-glycerophosphate, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, containing 1 mM dithiothreitol, 5 mM ATP, 1 μg of PHAS-1, and 10 mCi [γ-^{32}P]ATP. The products were analyzed by SDS–PAGE followed by autoradiography.

The Cdc2 kinase assay was performed for 30 min at 37° on the bead-bound immune complexes resuspended in 500 μL of 20 mM Tris, 4 mM $MgCl_2$, pH 7.4, containing 2.4 μg of histone H1, and 10 mCi [γ-^{32}P]ATP. The reaction was stopped by addition of an equal volume of Laemmli buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.5 mg/mL bromophenol blue), then the proteins were separated by 12% SDS–PAGE, and phosphorylated H1 protein visualized by autoradiography.

2.7. Statistical analysis

All data are the mean ± SD. Differences in cell cycle distribution were analyzed using the x^2 test. The differences between treated and control groups were analyzed by Wilcoxon signed rank test. Statistical analyses were performed using SAS (version 6.011; SAS Institute Inc.). A P value of <0.05 was considered statistically significant.

3. Results

3.1. Genistein arrest HepG2 cells at G2/M phase

To gain an initial insight into the effects of genistein on cell cycle distribution, the human hepatoma cell line, HepG2, was incubated for 24 hr with 0–30 μg/mL genistein. As shown in Fig. 1, increasing concentrations of genistein caused increasing accumulation of cells in G2/M phase.

3.2. Effects on cyclin B1 expression and Cdc2 activity and phosphorylation

To examine the expression of intracellular proteins regulating cell cycle progression at the G2/M boundary, cyclin B1 expression and Cdc2 kinase activity were analyzed. When cells were incubated for 24 hr with 0–20 μg/mL genistein, dose-dependent inhibition of Cdc2 kinase activity was seen (Fig. 2A), but there was no significant difference in cyclin B1 levels (Fig. 2B). Since the Cdc2-cyclin B1 complex is maintained in an inactive form by phosphorylation of residues Thr-14 and Tyr-15 on Cdc2, we examined Cdc2 levels and phosphorylation by Western blotting and found that both were increased after 24 hr of genistein treatment (Fig. 2C).

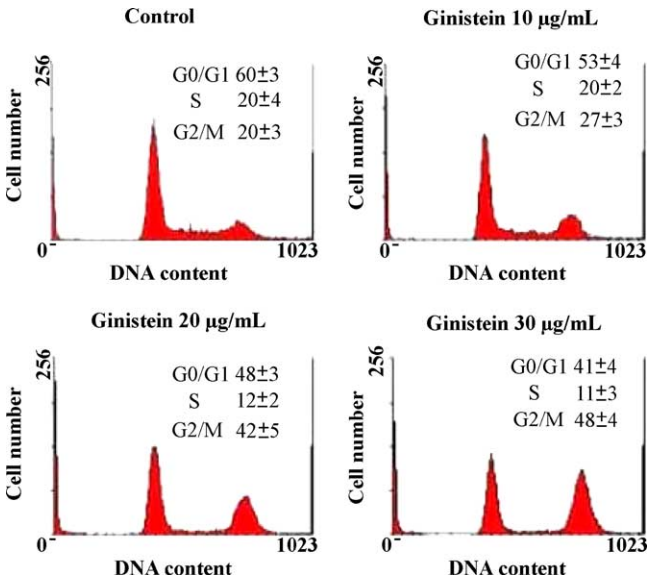


Fig. 1. Genistein arrests HepG2 cells at G2/M phase. HepG2 cells (1×10^6) were grown for 24 hr in the presence of 0, 10, 20, or 30 μg/mL genistein, then the cell cycle distribution was determined by flow cytometry. The main panel shows as single representative result, while the numerical values are the mean ± SD of at least three experiments.

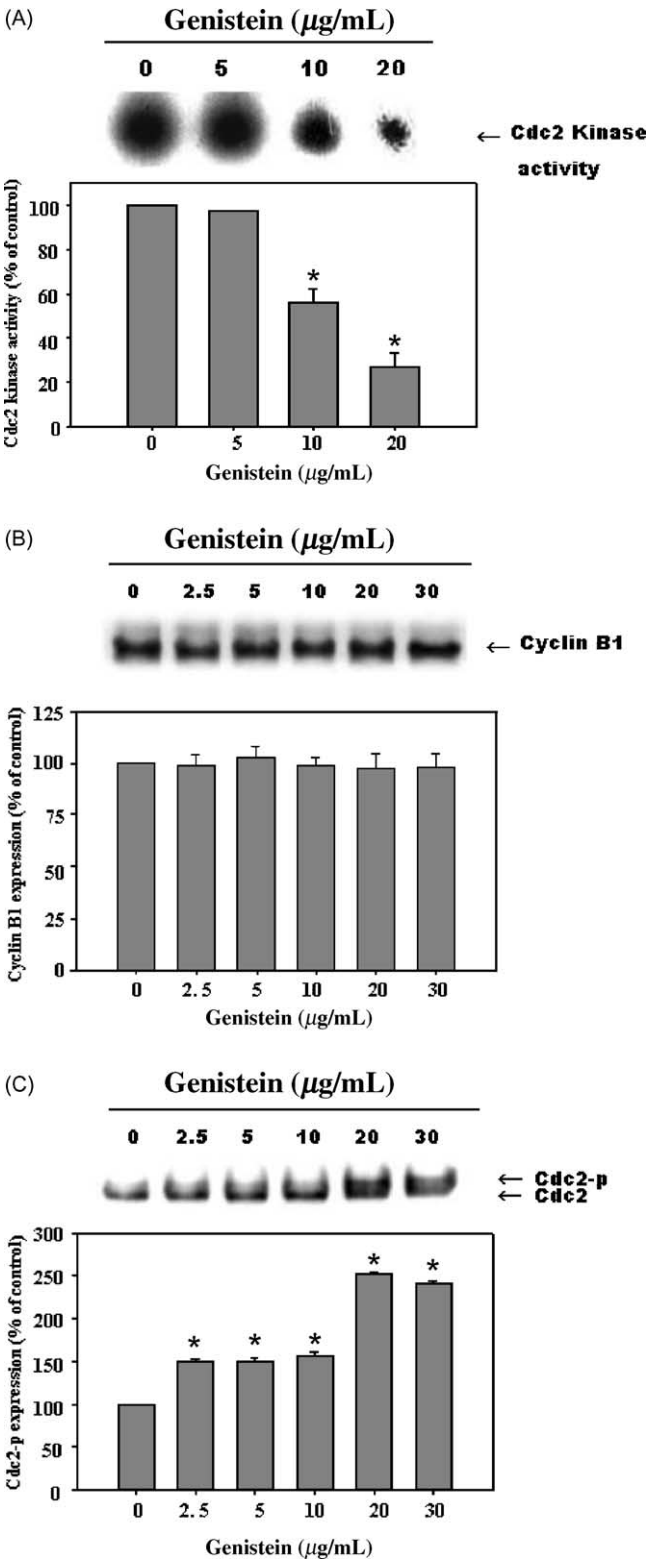


Fig. 2. Effect of genistein on Cdc2 kinase activity and cyclin B1 and Cdc2 protein levels. (A) HepG2 cells were incubated for 24 hr with 0, 5, 10, or 20 μg/mL genistein, then Cdc2 kinase activity measured by electrophoresis of the products on an SDS gel and autoradiography of phosphorylated H1 protein. (B and C) 2×10^6 HepG2 cells were treated for 24 hr with 0–30 μg/mL genistein, then levels of cyclin B1 (B) or Cdc2 were evaluated by Western blotting. The phosphorylated form of Cdc2 migrates slightly slower than the nonphosphorylated form, as indicated. The amount of the protein of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units of β-actin, then the density of the band was expressed as the relative density compared to that in untreated cells (control), taken as 100%. All experiments were performed at least three times and all data are the mean ± SD. $^*P < 0.05$ as compared with untreated group.

3.3. Genistein enhances Wee1 expression

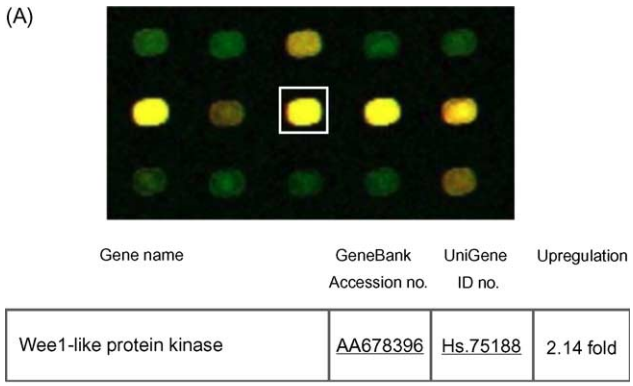
Thr-14 and Tyr-15 residues in the ATP-binding domain of Cdc2 are phosphorylated by Wee1 and dephosphorylated by the dual specificity phosphatase, Cdc25C. We therefore used microarray analysis to determine whether Wee1 mRNA levels were altered by genistein and found a 2.14-fold increase after 12-hr treatment with 20 μg/mL genistein (Fig. 3A). Western blotting showed that, after 24-hr treatment with 0–30 μg/mL genistein, Wee1 protein levels were also increased using 20 or 30 μg/mL genistein (Fig. 3B). These data suggest that increased *Wee1* gene expression contributes to the increased Cdc2 phosphorylation seen following genistein treatment.

3.4. Genistein increases $p21^{waf1/cip1}$ expression

Association of $p21^{waf1/cip1}$ with Cdc2-cyclin B1 complexes results in a decrease in Cdc2 activity. To determine whether $p21^{waf1/cip1}$ was involved in the genistein-induced reduction in Cdc2 activity, $p21^{waf1/cip1}$ gene and protein expression were analyzed. DNA microarray analysis showed that, after 12-hr treatment with 20 μg/mL genistein, $p21^{waf1/cip1}$ gene expression was increased 2.38-fold (Fig. 4A), while Western blots (Fig. 4B) of extracts of cells after 24-hr treatment with various concentrations of genistein showed a marked increase in $p21^{waf1/cip1}$ protein levels at genistein concentrations of 10–30 μg/mL, which was negatively correlated with Cdc2 activity (compare with Fig. 2A). These results suggest that induction of $p21^{waf1/cip1}$ expression might account for a large part of the reduction in Cdc2 activity.

3.5. Inactivation phosphorylation of Cdc25C by genistein

Cdc25C activates Cdc2 by dephosphorylation of residues Thr-14 and Tyr-15, which are phosphorylated by Wee1. Since *Wee1* gene expression was enhanced by genistein, we investigated the effect of genistein on Cdc25C levels and phosphorylation by Western blotting. As shown in Fig. 5, exposure of HepG2 cells to 0–30 μg/mL genistein for 24 hr showed that genistein concentrations of 20 and 30 μg/mL resulted in reduced Cdc25C expression, while the concentration of 30 μg/mL also resulted in increased Cdc25C phosphorylation on Ser-216. Since there was a decrease in Cdc25C expression in the absence of any increase in Cdc25C phosphorylation at 20 μg/mL genistein, a concentration leading to cell arrest, this suggests that Cdc25C inactivation was not only



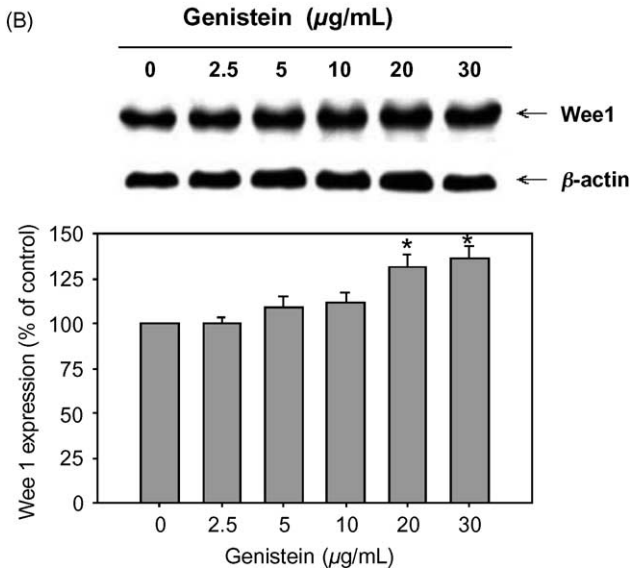
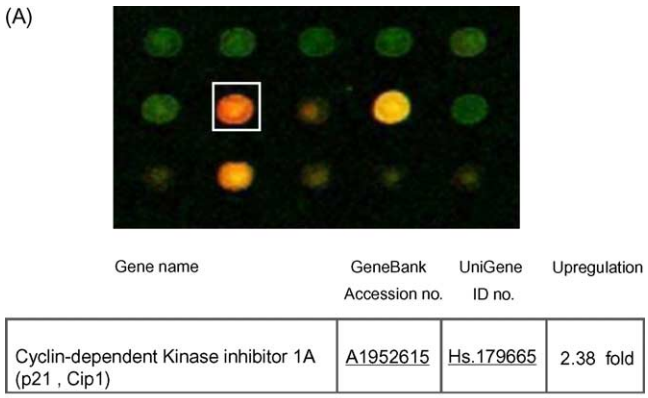


Fig. 3. Genistein increases Wee1 gene and protein expression. (A) 1×10^7 HepG2 cells were incubated for 12 hr with 20 μg/mL genistein, then total RNA was extracted and subjected to reverse-transcription to synthesize fluorescently labeled cDNA, which was then hybridized to microarrays spotted with target cDNAs prepared from existing gene libraries. The cDNA from the treated group was labeled with Cy5-dUTP (red) and that from the control group with Cy3-dUTP (green). The red spots represent transcripts upregulated in the treated group compared to the control group and the green spots represent downregulated transcripts. Yellow spots represent approximately equal levels of expression. (B) 2×10^6 HepG2 cells were incubated for 24 hr with 0–30 μg/mL genistein, then Wee1 protein levels were evaluated by Western blotting. The amount of the protein of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units of β-actin, then the density of the band was expressed as the relative density compared to that in untreated cells (control), taken as 100%. All experiments were performed at least three times and the data are expressed as the mean ± SD. $^*P < 0.05$ as compared with untreated group.



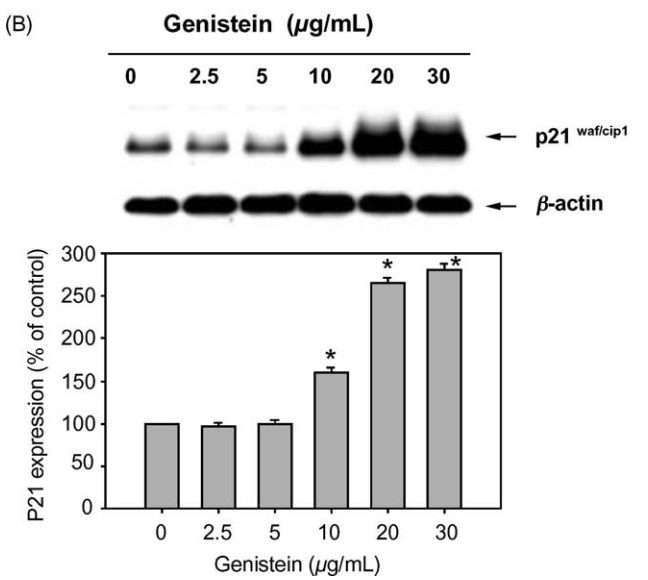


Fig. 4. Genistein increases $p21^{waf1/cip1}$ gene and protein expression. (A) The method used was as described in Fig. 3A examining $p21^{waf1/cip1}$ gene expression. (B) As in Fig. 3B, but estimating $p21^{waf1/cip1}$ protein levels. All experiments were performed at least three times and the data are expressed as the mean ± SD. $^*P < 0.05$ as compared with untreated group.

due to increased phosphorylation, but also to decreased nuclear export of active Cdc25C.

3.6. Genistein increases Chk2, p53, and $p21^{waf1/cip1}$ protein expression and the effect is inhibited by caffeine, an ATM kinase inhibitor

Checkpoint kinases can phosphorylate Cdc25C on Ser-216 by an ATM-dependent pathway [13,14]. To confirm that the inactivation of Cdc25C may due to ATM-dependent Chk2 activation, we examined the effects of genistein on the expression of Chk2 and p53, which are major targets of ATM kinase [24], and the effect of caffeine, an ATM kinase inhibitor [25,26], on these effects. When HepG2 cells were exposed to 0–30 μg/mL genistein for 24 hr, Chk2 levels showed a dose-dependent increase and the Chk2 band on Western blots showed a reduction in mobility (Fig. 6A), which has been shown to be due to phosphorylation [17]. A dose-dependent increase in p53 levels was also observed (Fig. 6B). When the effects of cotreatment with genistein and caffeine, an ATM kinase inhibitor, were tested on the above changes and on the increase in $p21^{waf1/cip1}$ protein levels (Fig. 4B), the effect of genistein on all three were inhibited (Fig. 6C), suggesting that genistein induces p53-dependent $p21^{waf1/cip1}$ expression and ATM-mediated Chk2 expression in hepatoma cells.

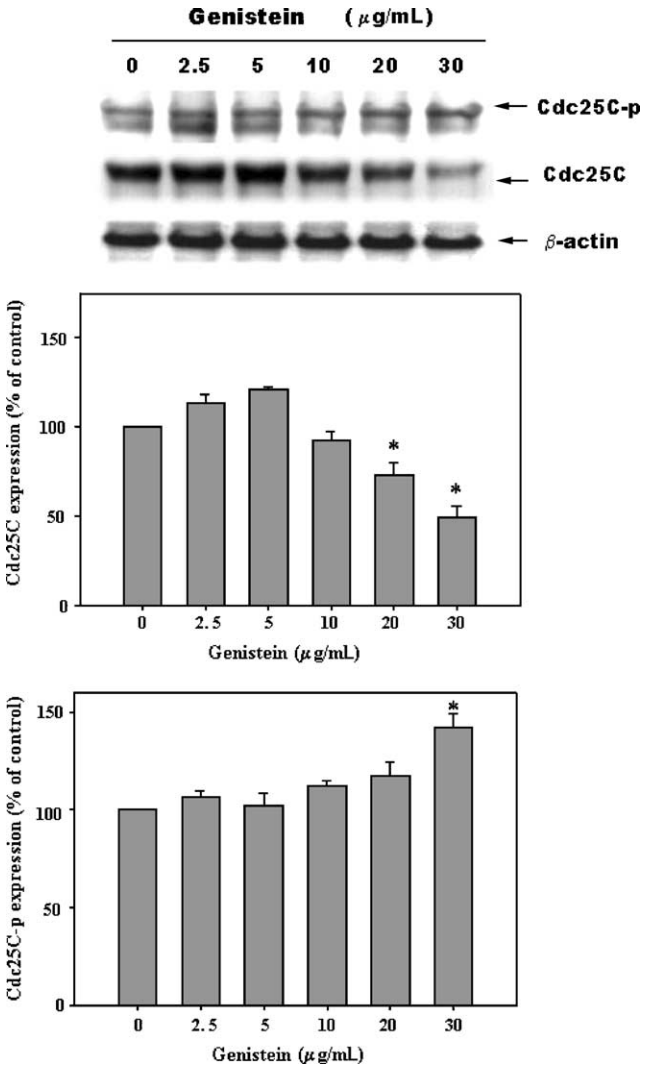


Fig. 5. Effect of genistein on Cdc25C levels and Ser-216 phosphorylation. 2×10^6 HepG2 cells were incubated for 24 hr with 0–30 μg/mL genistein, then levels of nonphosphorylated and phosphorylated Cdc25C were evaluated by Western blotting using polyclonal antibodies against human Cdc25C or Cdc25C-P, respectively. The amount of the protein of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units of β-actin, then the density of the band was expressed as the relative density compared to that in untreated cells (control), taken as 100%. All experiments were performed at least three times and the data are expressed as the mean ± SD. $^*P < 0.05$ as compared with untreated group.

3.7. Genistein enhances ATM kinase activity

When HepG2 cells were treated with 0–20 μg/mL genistein for 2 or 4 hr and ATM kinase activity assayed using the specific substrate, PHAS-1, ATM kinase activity was significantly increased after 2- or 4-hr treatment with 10 or 20 μg/mL genistein and after 4-hr treatment with 5 μg/mL genistein (Fig. 7).

4. Discussion

In this study, we found that genistein treatment decreased Cdc2 activity, increased Cdc2 phosphorylation, and enhanced the expression of the cell cycle inhibitor, $p21^{waf1/cip1}$. Furthermore, it increased both the levels of phosphorylated/inactivated Cdc25C phosphatase and Wee1 kinase. From these results, we conclude that genistein arrests HepG2 cells at G2/M phase (i) by inhibiting Cdc2 dephosphorylation/activation by causing a decrease in Cdc25C levels and an increase in Wee1 levels, and (ii) by decreasing Cdc2 activity by induction of expression of $p21^{waf1/cip1}$, which interacts with, and inhibits Cdc2. Genistein also activated the checkpoint kinase, Chk2, and increased p53 levels and ATM activity, and these effects were inhibited by caffeine, an ATM kinase inhibitor. Taken together, these results suggest that the effect of genistein on the levels of phosphorylated/inactivated Cdc25C are mediated, at least in part, by Chk2 activation *via* an ATM-dependent pathway.

ATM, the gene mutated in ataxia-telangiectasia, encodes a 370-kD protein which is a member of a family of proteins related to PI 3-K which have either lipid kinase or protein kinase activity. A subset of this family which shows greatest structural similarity to ATM is involved in DNA repair, DNA recombination, and cell cycle control [27]. In response to ionizing radiation-induced DNA damage, cells with wild-type ATM accumulate p53 protein and show increased p53 activity, whereas cells with defective ATM show a smaller increase in the amount of p53 protein [28]. Cells that lack ATM show delayed upregulation and phosphorylation of p53 [19] and defective phosphorylation of Chk2 in response to ionizing radiation [13]. *In vivo*, ATM, a manganese-dependent serine/threonine protein kinase, phosphorylates Ser-15 of p53 [28–30] and N-terminal sites in Chk2 [30] and is therefore a key player in signaling DNA damage to cell cycle checkpoints.

In the present study, we used Western blots to show that genistein increased p53 and Chk2 expression and that Chk2 mobility was reduced; this reduced mobility has been shown to be due to phosphorylation [24]. These effects of genistein were abrogated by caffeine, an ATM kinase inhibitor. The radiosensitizing effects of caffeine are due to inhibition of the protein kinase activities of ATM and the ATM- and Rad3-related kinase (ART) [31]. In this study, we showed that genistein increased ATM activity, but ART activity was not examined, so it is possible that other protein kinases that phosphorylate p53 and Chk2 are also activated by genistein treatment. Possible candidates include the related protein, DNA-PK, which phosphorylates Thr-68 of Chk2 and Ser-15 of p53 *in vitro* [30,32], and ART, which phosphorylates Ser-15 of p53 and Thr-68 of Chk2 *in vitro* [33]. Darbon *et al.* [31] showed that ATM is not involved in genistein-induced activation of p53 and Chk2, since treatment of cells with wortmannin, an inhibitor of PI 3-Ks, including ATM, ATR, and DNA-PK [28–30,34], did not inhibit genistein-induced phosphorylation of p53 and Chk2. In contrast, in our study, ATM was activated by genistein treatment, and caffeine, an ATM kinase inhibitor, reduced p53 and Chk2 expression.

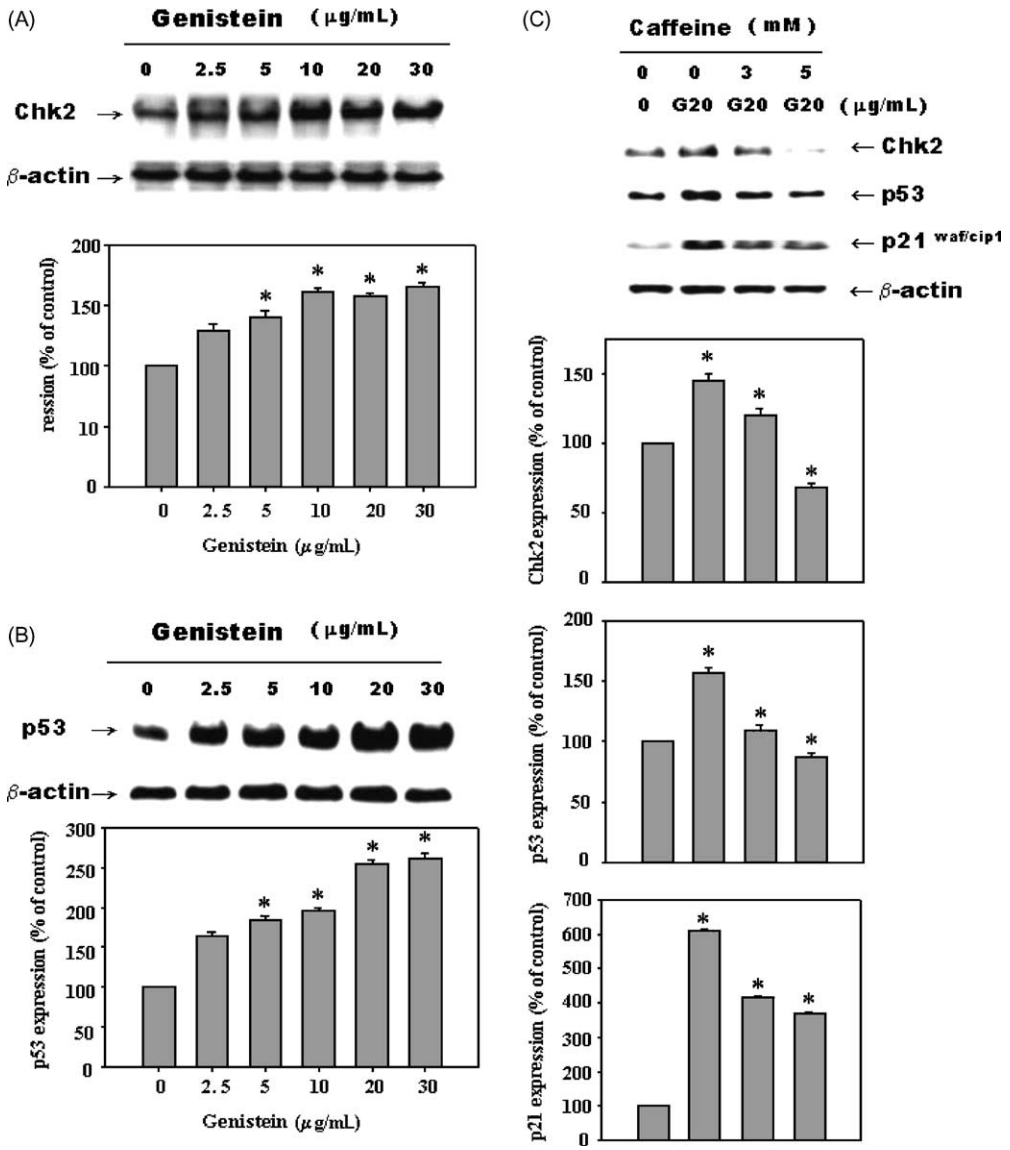


Fig. 6. Effects of caffeine on the genistein-induced increases in Chk2, p53, and $p21^{waf1/cip1}$ expression. 2×10^6 HepG2 cells were incubated for 24 hr with 0–30 μg/mL genistein (A and B) or with 20 μg/mL genistein and 0, 3, or 5 mM caffeine (C). Cell extracts were prepared, then assayed by Western blotting using antibodies against Chk2, p53, or $p21^{waf1/cip1}$. The amount of the protein of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units of β-actin, then the density of the band was expressed as the relative density compared to that in untreated cells (control), taken as 100%. All experiments were performed at least three times and the data are expressed as the mean ± SD. $^*P < 0.05$ as compared with untreated group.

We speculate that these differences may be due to the different cell lines used in the two studies.

p53 protein plays a critical role in regulating cell cycle progression after DNA damage. The mechanism by which it mediates cell cycle arrest at the G1 checkpoint involves transactivation of the cyclin-dependent kinase inhibitor, $p21^{waf1/cip1}$ [35]. Recently, it was shown that a novel form of $p21^{waf1/cip1}$ protein can inhibit growth by acting not at G1, but at G2/M [22]. In addition, $p21^{waf1/cip1}$ can associate with the activated Tyr-15 dephosphorylated form of Cdc2, and this complex is devoid of kinase activity, indicating that $p21^{waf1/cip1}$ may play a role in Cdc2 inhibition and G2 arrest [36]. It has been reported that $p21^{waf1/cip1}$ expression is rarely p53-independent, e.g. $p21^{waf1/cip1}$ expression is blocked in cells from p53 knockout mice [37]. However, p53-independent $p21^{waf1/cip1}$ expression is induced in antioxidant-treated colorectal cancer cells [38]. Since our results showed that, in HepG2 cells, genistein induced p53 accumulation and upregulated $p21^{waf1/cip1}$ expression and that both effects were attenuated by caffeine, we speculate that p53-dependent $p21^{waf1/cip1}$ expression might occur in genistein-treated HepG2 cells. However, p53-independent $p21^{waf1/cip1}$ expression cannot be excluded, because the attenuating effects of caffeine on genistein-mediated $p21^{waf1/cip1}$ expression and p53 accumulation seem to be quantitatively different, the former being affected to a greater degree. However, further studies are required to determine whether p53-independent $p21^{waf1/cip1}$ expression is induced in genistein-treated HepG2 cells.

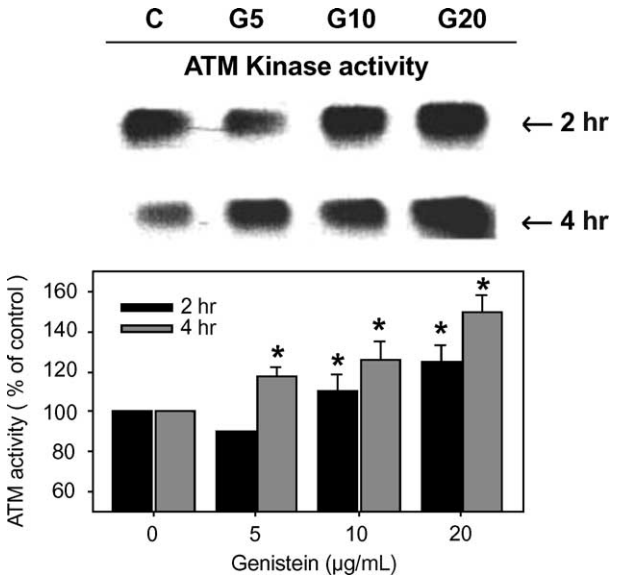


Fig. 7. Genistein increases ATM kinase activity in HepG2 cells. 2×10^6 cells were incubated for 2 or 4 hr with 0, 5, 10, or 20 μg/mL genistein, then ATM was immunoprecipitated and kinase activity assayed using PHAS-1 as substrate, followed by SDS–PAGE and autoradiography. $^*P < 0.05$ as compared with untreated group.

Leach *et al*. [39] reported that p53 downregulates Wee1 expression, resulting in Cdc2 dephosphorylation and the overriding of an important cellular checkpoint that protects against apoptosis. However, our results showed that Wee1 expression was upregulated by genistein treatment, despite the simultaneous increase in p53 accumulation. This suggests that other pathways may be involved in the upregulation of Wee1 expression and that these overcome p53-mediated downregulation. Sty1/Spc1 MARK-dependent transcriptional and Sty1/Spc1 MARK-independent post-transcriptional regulation are involved in regulating Wee1 expression [40]. Further studies are required to clarify whether the MARK-dependent and/or MARK-independent pathway is involved in genistein-induced Wee1 upregulation in HepG2 cells. As discussed above, we propose that genistein arrests HepG2 cells at G2/M phase by ATM-mediated Chk2 expression and upregulation of $p21^{waf1/cip1}$ and Wee1 expression (Fig. 8).

The *in vitro* effective concentration of genistein (10–30 μg/mL) for G2/M arrest of hepatoma cells in this study was similar to that for other cancer cells [8,41], but higher than that needed for non-neoplastic cells [42,43]. Peterson *et al*. [44] have reported that normal mammary epithelial cells are more responsive than MCF-7 breast cancer cells to genistein treatment, presumably because of altered genistein metabolism. Interestingly, Frey *et al*. [42] reported that genistein-induced cell cycle arrest is not associated with apoptosis in MCF-10F cells, and our previous study [7] also showed that genistein-induced G2/M cell cycle arrest in hepatoma cells is not a prerequisite for apoptosis and that addition of caffeine to genistein-treated hepatoma cells not only prevented the accumulation of cells in G2/M phase, but also increased the percentage of apoptotic cells, the IC_{50} for growth inhibition being lower than that for cell cycle arrest. Based on these observations, it is likely that the antiproliferative mechanism of genistein may not be related to the mechanism required for cell cycle arrest and that the effects on cell

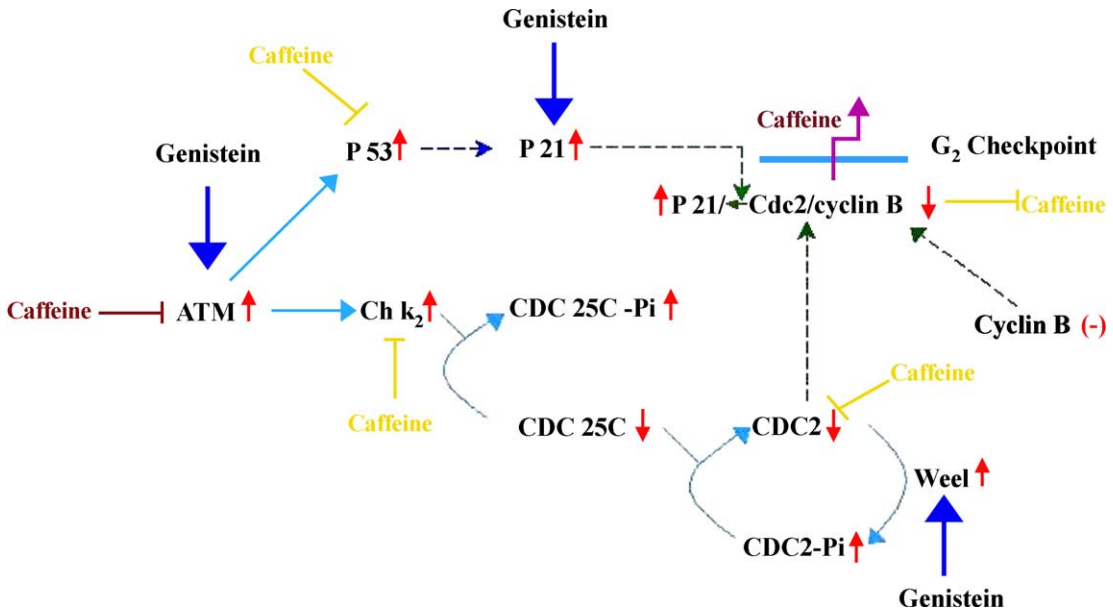


Fig. 8. Schematic diagram of the effects of genistein on HepG2 cells. Genistein treatment increases both Cdc2 phosphorylation and expression of the Cdc2-active kinase, Wee1. In addition, levels of $p21^{waf1/cip1}$, which interacts with Cdc2, are increased. Phosphorylation/inactivation of Cdc25C, which dephosphorylates/activates Cdc2, is also increased. Genistein also induces p53 accumulation, increases ATM activity, and activates the checkpoint kinase, Chk2, which phosphorylates/inactivates Cdc25C. Caffeine, an ATM kinase inhibitor, blocks the effects of genistein on Chk2, p53, and $p21^{waf1/cip1}$. (↑) increased; (↓) decreased; (|) abolished.

cycle arrest may be an ''epiphenomenon''. G2/M phase arrest is seen in cells exposed to a variety of DNA-damaging agents and may allow the cells time for DNA repair. Accordingly, the cell cycle arrest effect of genistein could be helpful in the treatment of chemically induced carcinogenesis. There is growing evidence that ablation of G2 checkpoint function in human tumor cell lines increases their sensitivity to several anticancer agents [45], and a drug, like UCN-01, which overrides a checkpoint in cancer cells, could potentially be useful in combination cancer therapy along with radiation or other DNA-damaging agents [45]. This raises the question whether cell cycle arrest by genistein attenuates the effects of some anticancer agents. Genistein has been reported to have a tumor enhancing effect [46–48]. Since genistein has divergent biological function, additional studies will be needed to evaluate the concentration range of dietary genistein required to overcome the inhibitory effect of other anticancer agents on tumor growth and to understand the molecular mechanisms of the interaction between genistein and other agents. In addition, data are required on the bioavailability of genistein and its metabolism in the liver and the exposure of hepatomas to genistein, but great care should be taken with the amount of genistein administered.

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