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p53-independent Induction of p21 (WAF1/CIP1), Reduction of Cyclin B1 and G2/M Arrest by the Isoflavone Genistein in Human Prostate Carcinoma Cells

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Genistein, a natural isoflavonoid phytoestrogen, is a strong inhibitor of protein tyrosine kinase and DNA topoisomerase II activities. Genistein has been shown to have anticancer proliferation, differentiation and chemopreventive effects. In the present study, we have addressed the mechanism of action by which genistein suppressed the proliferation of p53-null human prostate carcinoma cells. Genistein significantly inhibited the cell growth, which effect was reversible, and induced dendrite-like structure. The inhibitory effects of genistein on cell growth proliferation were associated with a G2/M arrest in cell cycle progression concomitant with a marked inhibition of cyclin B1 and an induction of Cdk inhibitor p21 (WAF1/CIP1) in a p53-independent manner. Following genistein treatment of cells, an increased binding of p21 with Cdk2 and Cdc2 paralleled a significant decrease in Cdc2 and Cdk2 kinase activity with no change in Cdk2 and Cdc2 expression. Genistein also induced the activation of a p21 promoter reporter construct, utilizing a sequence distinct from the p53-binding site. Analysis of deletion constructs of the p21 promoter indicated that the response to genistein could be localized to the 300 base pairs proximal to the transcription start site. These data suggest that genistein may exert a strong anticarcinogenic effect, and that this effect possibly involves an induction of p21, which inhibits the threshold kinase activities of Cdks and associated cyclins, leading to a G2/M arrest in the cell cycle progression.

Key words: Genistein — Cell cycle — G2/M arrest — p21

Epidemiological data have demonstrated that diet may be the most important environmental factor involved in the etiology of some of the most prevalent forms of cancer.[1)] Therefore natural products have been a good source of novel chemotherapeutic compounds. Soy consumption has been reported as a candidate to reduce the risk of acquisition of cancer.[2)] Several compounds in soybeans have demonstrated anti-cancer activity. One of the candidates compounds against malignancy in soybeans is genistein (4′,5,7-trihydroxyisoflavone), the most abundant isoflavone present in soybeans and soy-based products, which was originally isolated from fermentation broth of *Pseudomonas* spp.[3)] Several mechanisms have been proposed for the effects of genistein. Akiyama *et al*.[3)] were the first to describe the ability of genistein to inhibit specifically the protein tyrosine kinase (PTK) activity. PTKs seem to play a key role not only in tumorigenesis, but also in differentiation.[4)] They are also known to be associated with both growth control receptors[5)] and several oncogene products.[6)] These data suggested that genistein may have important anticancer properties. Other investigators have suggested that genistein may exert its effect through inhibition of DNA topoisomerase II[7)] and ribosomal S6 kinase activities,[8)] which may lead to protein-linked DNA strand breaks.[9)] In addition, genistein has been observed to act *in vitro* as a potent inhibitor of angiogenesis[10)] and of the development of metastases.[11)] *In vivo* studies also demonstrated that genistein is effective in reducing tumorigenesis and carcinogenesis.[12, 13)] Although a specific mechanism of action has not been identified, previous studies have shown that genistein inhibited the growth of wide range of cultured cancer cells,[14–19)] induced differentiation of several malignant cell lines,[9, 17, 20)] and caused apoptotic cell death.[14, 15, 18, 21)] To date, despite these accumulated data, the molecular mechanism of its antiproliferative action on cancer cells is poorly understood.

Progression through the cell cycle is regulated by sequential activation and subsequent inactivation of a series of cyclin-dependent kinases (Cdks).[22, 23)] The activities of Cdks are positively regulated by cyclins, which act at different checkpoints of the cell cycle. The D-type cyclins and cyclin E are required for progression through G1. As cells enter G1, the cyclin D/Cdk4 (and/or Cdk6) complex appears to be necessary for transition through early G1,[24, 25)] whereas cyclin E/Cdk2 complex is required in transition from late G1 into S phase.[26, 27)] Cyclin A is produced in late G1 and it accumulates during S and G2

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phase, while expression of B-type cyclins is typically maximal during the G2 to M phase transition and controls passage through the M phase. Cyclin A associates with and activates primarily Cdk2,[28, 29] whereas B-type cyclins are the major activators of Cdc2 (also called as Cdk1).[30, 31] The activity of Cdks is negatively regulated by binding to Cdk inhibitors in response to a variety of antiproliferative signals.[32, 33] Among several Cdk inhibitors, p21 (WAF1/CIP1), the archetypal member of this family, which was isolated as a Cdk2-associated protein and an inhibitor of Cdk2,[34, 35] is an important mediator of cell cycle arrest imposed by the tumor suppressor p53 in response to DNA damage.[36–39] Recent studies implicate growth arrest accompanied by up-regulation of p21 not only in inhibiting proliferation, but also in promoting differentiation.[37, 40] p21 is also known to be an inhibitor of DNA replication by inhibiting proliferating-cell nuclear antigen, which is also required for cell cycle progression.[41] In addition to being induced by p53, p21 is also induced by other factors in the p53-independent pathway.[38, 40, 42–45] Several studies have demonstrated that relative levels of p21 may be critical in determining the threshold kinase activity of various cyclin/Cdk complexes,[32, 33, 35–37, 46] suggesting that appropriate levels of p21 may be critical in the regulation of cell growth.

In the present study, we examined the effect of genistein on the growth of human prostate carcinoma PC-3-M cells that lack wild-type p53. The present results demonstrated that genistein was able to inhibit growth of cells and induced morphological changes, which effects were reversible. Genistein treatment resulted in arrest at the G2/M check point of the cell cycle, which was related to up-regulating the Cdk-inhibitor p21, and down-regulating the intracellular levels of cyclin B1. This study also showed that the up-regulation of p21 was associated with enhanced binding of p21 with Cdk2 and Cdc2. In addition, using deletion constructs of the p21 promoter, we found that the response to genistein could be localized to the 300 base pairs proximal to the transcription start site. Therefore we suggest that genistein inhibits cell growth by inducing p53-independent transcriptional regulation of p21, leading to down-regulation of the activity of Cdk2 and Cdc2 kinase activity.

MATERIALS AND METHODS

Cell culture and treatments Human PC-3-M prostate carcinoma cells which are a metastatic variant of PC3 cells, as described by Kozlowski *et al*.,[47] were grown in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin and streptomycin (Biofluids, Rockville, MD) in a humidified 5% CO_2 incubator at 37°C. Genistein (Sigma Chemical Co., St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO, Sigma) for all experiments. The final DMSO concentration did not exceed 0.05%.

Growth study and morphology For growth inhibition analysis, cells were plated at 0.5×10^4 cells per 10-mm plate, and incubated for 24 h. Cells were cultured in the presence or absence of variable concentrations of genistein in culture medium enriched with 10% FBS. After 72 h of culture, cells were trypsinized and the viable cells were scored using the Trypan Blue method. For the morphological study, cells were grown on coverslips and treated with 100 μM genistein for 36 h. Cells were Wright-stained (Fisher Scientific, Pittsburgh, PA), as recommended by the manufacturer, and photographed.

DNA flow-cytometric analysis Cells were harvested by gentle scraping at indicated time points, pelleted by low-speed centrifugation, resuspended in 200 μl of citrate buffer (250 m*M* sucrose, 40 m*M* trisodium citrate, 5% Me_2SO, pH 7.6), and frozen at −80°C. Before staining, cells were thawed quickly and treated with RNase A (0.1 mg/ml). Nuclei were stained with propidium iodide (PI). All solutions were prepared in a stock solution containing 3.4 m*M* trisodium citrate, 0.1% NP-40, 1.5 m*M* spermine×4 HCl, 0.5 m*M* Tris-base, pH 7.6. DNA content in each cell nucleus was determined in a FACScan flow cytometer (Becton-Dickinson, San Jose, CA).

Immunoprecipitation and western blot analysis Total cell lysates were incubated for 1 h at 4°C with anti-Cdk2 or anti-Cdc2 antibody. The immuno-complex was collected on protein A-Sepharose beads (Sigma) for 1 h and washed 5 times with TNN buffer [40 m*M* Tris (pH 8.0), 120 m*M* NaCl, 0.5% NP-40, 0.1 m*M* sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 100 μg/ml phenylmethylsulfonyl fluoride] prior to boiling in sodium dodecyl sulfate (SDS) sample buffer. Immunoprecipitated proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Western blot analysis was performed as described.[19] Monoclonal antibodies to Cdc2, cyclin A, cyclin B1 and polyclonal antibody to Cdk2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal anti-p21 antibody was obtained from Transduction Lab. (Lexington, KY). Monoclonal antibody to p53 was purchased from Calbiochem (Cambridge, MA). Peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham Corp. (Arlington Heights, IL).

Immune-complex kinase assay Cell lysates from untreated control and genistein-treated cells were incubated with primary antibody for 1 h at 4°C. Immuno-complexes were collected on protein A-Sepharose beads. The beads were washed three times with TNN buffer and three times in reaction buffer [20 m*M* Tris-HCl (pH 7.5), 4 m*M* $MgCl_2$]. The beads were then resuspended in reaction

buffer with [γ-^{32}P]ATP (adenosine triphosphate, ICN, Irvine, CA) and histone H1 (Sigma) as a substrate. After incubation at 37°C for 30 min, the reaction was stopped by the addition of the same amount of 2× SDS sample buffer. After boiling and spinning, the samples were separated on 10% SDS-polyacrylamide gels. The gels were dried, and bands were detected by autoradiography.

Northern blot analysis Total cellular RNA was extracted using an RNAzol B RNA isolation kit (Tel-Test, Inc., Friendswood, TX), following the procedure described by the manufacturer. Twenty micrograms of total cellular RNA was denatured, electrophoresed on 1% agarose-formaldehyde gels and blotted onto nylon hybridization membranes (Hybond-N, Amersham Corp.). After prehybridization, membranes were hybridized overnight with an excess of ^{32}P-labeled (ICN) cDNA probes of p21, and then washed under highly stringent conditions. The hybridized membrane was exposed to X-ray film for 2 days.

p21 promoter-luciferase constructs and transfection assay Three constructs, the full-length 2.4-kb human p21 promoter constructs as well as p21 promoter deletion constructs, in which 300 base pairs were progressively removed from the 3′ terminus, were tested as follows: a 2.4-kb p21 promoter fragment containing the p21 transcription initiation start site at its 3′ end and two p53 responsive element at the 5′ end, a 1.8-kb fragment consisting of the 2.4-kb fragment from which 600 base pairs, including a p53 response element, had been deleted from the 5′ end, and a 0.3-kb fragment consisting of the 300 base pairs most proximal to the transcription start site.[39, 45)]

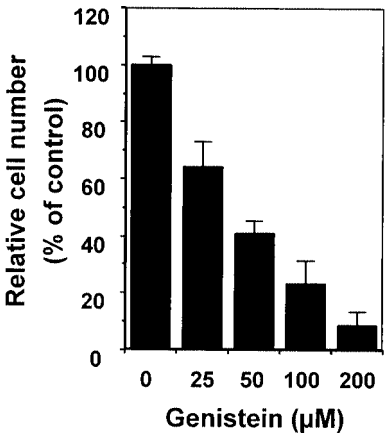

Fig. 1. Inhibition of cell proliferation by genistein in PC-3-M cells. From 24 h after inoculation, cells were cultured for 72 h in the absence or presence of genistein at concentrations ranging from 25 to 200 μM, and the number of viable cells was counted with a hematocytometer. Results are the percentage of viable cells with respect to controls incubated under the same conditions. Data represent means of triplicate experiments.

Cells were transiently transfected with p21 promoter-luciferase reporter constructs using LipofectAMINE (Gibco BRL), as recommended by the manufacturer. Following transfection, the cells were incubated for 12 h, the medium was exchanged, and the cells were incubated for an additional 36 h in the presence or absence of 100 μM genistein. The cells were then lysed, and luciferase activity in the lysates was assayed using a Dynatech ML1000 luminometer (Dynatech Laboratories, Chantilly, VA).

RESULTS

Genistein inhibits cell proliferation To evaluate the effects of genistein on cell proliferation of human prostate carcinoma cell line PC-3-M, we initially assessed the effect of this drug on the growth of PC-3-M cells. After seeding, cells were cultured for 72 h with increasing concentrations of genistein, and cells were scored with a hematocytometer. As shown in Fig. 1, genistein caused a strong concentration-dependent inhibition of PC-3-M cell growth. On day 3, the growth of cells was inhibited to 58, 77 or 90% of the control by genistein at 50, 100 or 200 μM, as was observed in various prostate cancer cell lines.[16)] In cologenic assays, there was no marked decrease in cologenicity after genistein treatment. This assay, in which cells were treated with genistein for 24 h, the genistein-contained medium was washed out, and the cells were recultured in complete medium in the absence of genistein, demonstrates that after 24 h incubation the effect of genistein was reversible (data not shown).

Genistein induces morphological change It was reported[17, 20)] that genistein-induced differentiation involved an increased melanin content and dendrite-like structure formation in metastatic melanoma cells. We therefore examined whether genistein induced the differentiation of PC-3-M cells. Cells were treated with genistein, Wright-stained, and analyzed for changes in morphology. As can be seen in Fig. 2, control cells had an epithelial morphology and tended to grow in clusters with a somewhat acinar appearance. In contrast, PC-3-M cells developed features of neuronal morphology after addition of genistein. Genistein-treated cells had a neuronal appearance, characterized by bipolar or multipolar cells with long processes and beaded varicosities. These dendritic structures became progressively longer, and by 72 h after treatment, many cells showed an extensive network of such structure. Equivalent morphologic effects were seen with lower doses of genistein (data not shown).

Genistein induces G2/M arrest To identify if the suppressive effect is caused by specific perturbation of cell cycle-related events, we next measured the DNA content of untreated control and genistein-treated PC-3-M cells using flow cytometry after PI staining of nuclei. As shown in Fig. 3, the population of G1 and S cells continuously

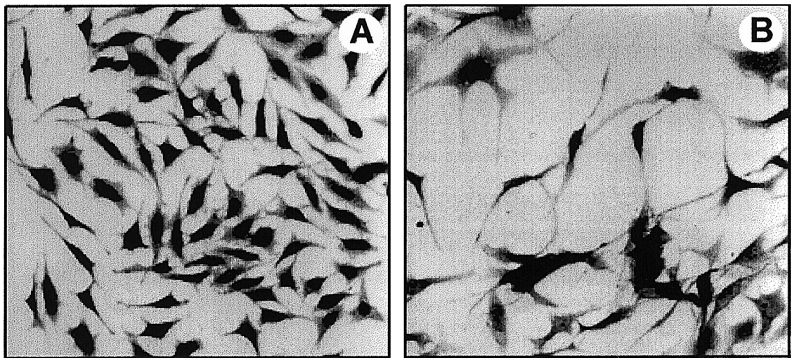

Fig. 2. Morphological changes in PC-3-M cells after exposure to genistein. Exponentially growing cells were incubated with either vehicle alone (A) or 100 μM genistein for 36 h (B), and Wright-stained. Cell morphology was visualized by light microscopy. Magnification, ×20.

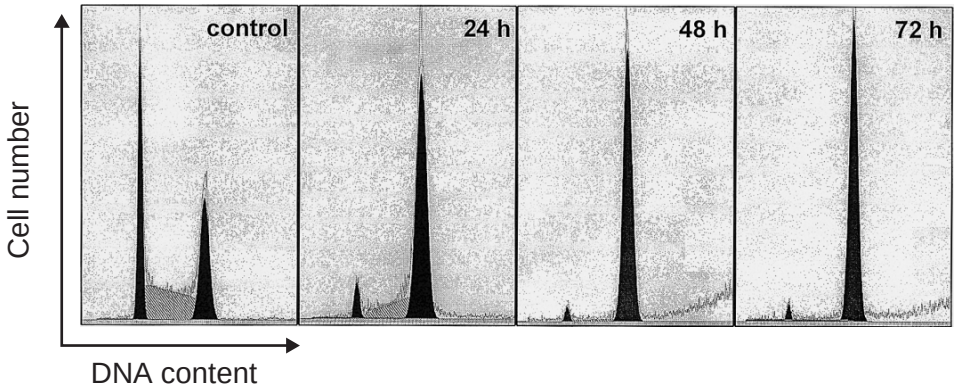

Fig. 3. DNA-fluorescence histogram of PC-3-M cell nuclei after genistein treatment. Exponentially growing cells at 60–70% confluency were treated at 0 time with 100 μM genistein. Cells were trypsinized and pellets were collected. The cells were fixed and digested with RNase, and then cellular DNA was stained with PI, and analyzed by flow cytometry at different time points after genistein exposure.

decreased in a time-dependent manner after exposure to genistein. At 24 h after addition of 100 μM genistein, there was a 2.9-fold increase in the percent of PC-3-M cells in G2/M. By 48 h following genistein treatment, more than 90% of cells were arrested in the G2/M phase of the cell cycle, while very few G1 cells were observed. These results suggested that PC-3-M cells were arrested by genistein at the G2/M phase of the cell cycle, as was observed in several cancer cell lines.[14–19] To elucidate the reversibility of the effect of genistein on cell cycle progression, cells were incubated with genistein for 24 h, then the medium was changed to a fresh one without genistein and culture was continued for an additional 24 h. The effect of G2/M arrest of the cell cycle by genistein was reversible 24 h after washing of the cells which had been exposed to genistein (data not shown).

Genistein inhibits expression of cyclin B1 Eukaryotic cell cycle progression is regulated by a series of Cdks and cyclins at different phases. Since genistein treatment perturbed the G2/M phase of the cell cycle, as assessed by DNA flow cytometry, we examined the expression of intracellular proteins of the cell cycle regulating components at the G2/M boundary, such as cyclin A, cyclin B1, Cdk2 and Cdc2, whose expression is induced in cells entering M from G2, in response to genistein treatment. As compared to the untreated control, western blot analysis of the samples obtained after genistein treatment for different time periods showed no significant change in the intracellular protein levels of cyclin A, Cdk2 and Cdc2 (Fig. 4A). On the other hand, genistein treatment resulted in a time-dependent decrease in the levels of cyclin B1, a protein which plays an important role in the positive regu-

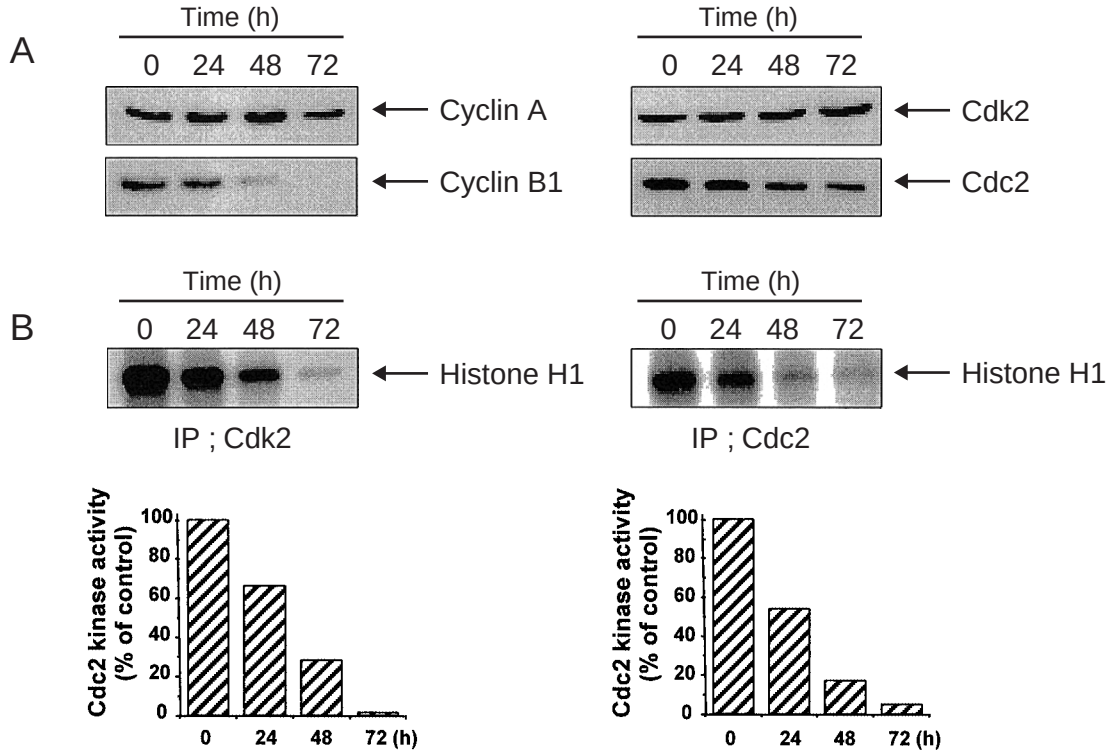

Fig. 4. Effect of genistein on the expression of cyclins and Cdks protein, and Cdks kinase activity in PC-3-M cells. Cells were treated with 100 μM genistein for the time indicated. A. Total cell lysates were prepared, and equal amounts of protein lysates were electrophoretically separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. Western blots were detected with antibodies against cyclin A, cyclin B1, Cdk2 and Cdc2, and enhanced chemiluminescence (ECL) detection. B. Total cell lysates were prepared and immunoprecipitated with anti-Cdk2 or anti-Cdc2 antibody, and kinase activity was assayed using histone H1 as a substrate. Phosphorylated histone H1 band intensity was quantitated and normalized with respect to the control for each treatment.

lation of Cdc2 activity at the G2/M phase, which is consistent with the effective times for significant induction of G2/M arrest of the cell cycle. It is suggested that the genistein-induced growth arrest in human prostate carcinoma PC-3-M cells is partially due to its down-regulating effect on the intracellular levels of cyclin B1, as was observed in breast carcinoma cells.[19)]

Genistein inhibits Cdk2 and Cdc2 kinase activity Cdc2 kinase activity, which is activated by cyclin B1, is essential to G2/M progression, and cyclin A binds to and activates the Cdk2 and Cdc2 protein kinases during the S and G2 phases.[28–31)] Genistein treatment perturbed the G2/M phase of the cell cycle, but did not affect the protein levels of Cdk2 and Cdc2. We then examined whether genistein inhibits the Cdk2 and Cdc2 kinase activities with the immuno-complexes that had been immunoprecipitated with antibodies to Cdk2 and Cdc2 from the cell lysates treated with genistein. As shown in Fig. 4B, the Cdk2 kinase activity using histone H1 as a substrate was significantly inhibited in a time-dependent manner in response to genistein treatment. At 48 h and 72 h after treatment of genistein, decreases of as much as 70% and 98%, respectively, in Cdk2 kinase activity were observed. Similarly, genistein treatment caused a 78% and 94% reduction in Cdc2 kinase activity, relative to the control. These findings also agreed well with the effective times of genistein for induction of cell cycle arrest and inhibition of the expression of cyclin B1 protein. These data indicated that genistein inhibited Cdks kinase activity by changing the activation states, rather than altering the expression, of Cdks proteins.

Genistein induces p53-independent expression of p21 protein and mRNA To understand the molecular mechanism by which genistein down-regulates the Cdk2 and Cdc2 kinase activities, we next investigated whether Cdk inhibitors, such as p21 and p27, are involved in the genistein-induced growth arrest in PC-3-M cells by western and northern blot analysis (Fig. 5A). In the untreated control cells, expression of p21 at the protein and mRNA levels was undetectable. However, incubation of cells with

genistein caused a striking time-dependent increase in the magnitude of induction of p21 protein and mRNA between 24 to 48 h, whereas p27 was not detectable in PC-3-M cells without or with genistein treatment (data not shown). The induction of p21 expression is consistent with the result that genistein completely inhibited the growth and cell cycle progression of PC-3-M cells after 48 h treatment. As *p53* gene is deleted in PC-3-M cells, it is most likely that the induction of the p21 is mediated in a p53-independent fashion.

Genistein induces association of p21 with Cdk2 and Cdc2 The above results clearly suggest that the down-regulation of Cdk2 and Cdc2 kinase activities by genistein is largely caused by an inductive effect on the level of p21

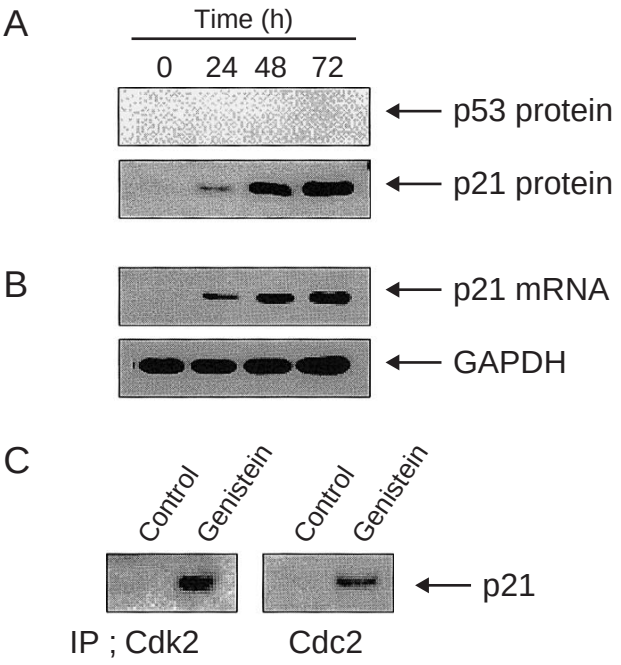

Fig. 5. Induction of cyclin-dependent kinase inhibitor p21 expression, and association of p21 with Cdks in genistein-treated PC-3-M cells. A. PC-3-M cells were incubated with 100 μM genistein for the time indicated. Equal amounts of total cell lysates were subjected to 12% SDS-polyacrylamide gels and immunoblotting, and the membranes were probed with antibodies to p53 and p21. B. Equal amounts of total RNA were loaded in each lane and separated by formaldehyde denaturing agarose gel electrophoresis. Northern blots were probed with p21 cDNA. The same blot was probed with a cDNA to glyceraldehyde-3′-phosphate dehydrogenase (GAPDH) to confirm loading of comparable levels of mRNA per lane. C. After treatment with genistein for 48 h, total cell lysates were immunoprecipitated with anti-Cdk2 or anti-Cdc2 antibody, separated on 12% SDS-polyacrylamide gels, and transferred to nitrocellulose. p21 was quantitated with anti-p21 antibody and ECL detection.

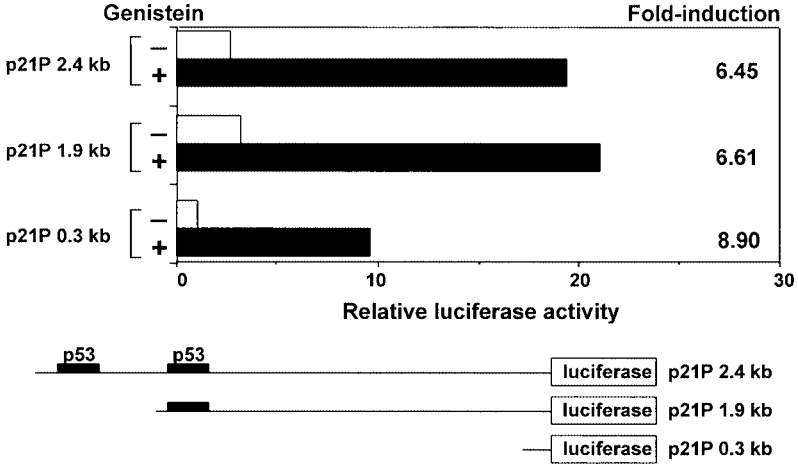

Fig. 6. Regulation of the transcriptional activity of the p21 promoter by genistein. The p21 promoter constructs fused to the luciferase gene were transiently transfected into PC-3-M cells, and incubated for 6 h. The medium was changed, and the cells were further incubated for 36 h in the presence of 100 μM genistein. The results are expressed as the mean±the standard deviation of data from three separate samples.

expression. It is well known that p21 inhibits the activity of Cdks by direct association with various cyclin/Cdk complexes, so that the complex formation of cyclins/Cdks/p21 is increased in cells arrested by DNA-damaging agents.[36, 37, 39, 40] We therefore investigated whether the observed decrease in Cdks kinase activity was due to an increase in binding to the induced p21 protein following genistein treatment. As shown in Fig. 5B, association of p21 with Cdks was almost undetectable by co-immunoprecipitation analysis of the untreated log-phase cells. However, treatment of the cells with genistein resulted in a significant increase in the binding of Cdk2 and Cdc2 with p21. These results demonstrate that the down-regulation of Cdk2 and Cdc2 kinase activities by genistein treatment is mainly caused by highly induced p21 expression.

Genistein stimulates p21 promoter activity Since the p21 expression is markedly induced by genistein in PC-3-M cells, we next investigated whether the up-regulation of p21 expression by genistein involves transcriptional regulation of the *p21* gene promoter. PC-3-M cells were transiently transfected with wild-type p21 promoter-luciferase fusion plasmids and luciferase activity was measured in untreated control cells and cells treated with genistein. Following 12 h exposure to genistein, the full-length 2.4-kb promoter construct containing two p53 response elements was activated 2.9-fold by genistein compared to the control. Luciferase activity was increased 3.2- and 6.45-fold in cells harvested 24 h and 36 h after genistein treatment, respectively. This time-response study using wild-type p21 promoter is consistent with the results that genistein inhibited the growth of PC-3-M cells and induced p21 protein and mRNA expression. Next, we tried to determine what regions of the p21 promoter are responsive to genistein activation. For this purpose, two 5′ deletion constructs of the p21 promoter were compared with wild-type p21 promoter (Fig. 6). After transfection into PC-3-M cells, luciferase activities following genistein treatment for 36 h were measured. A 1.9-kb construct lacking a p53 response element was activated by genistein to the same extent as the full-length promoter. The same level of inducibility was seen when cells were transiently transfected with a 0.3-kb construct consisting of 300 base pairs most proximal to the transcription start site. This result suggests that the genistein-responsive site is localized in the promoter region within 300 base pairs of the start site of transcription. Furthermore, it is considered that the p53 binding sites of the p21 promoter are not required for the transcriptional activation by genistein, since the promoter region of the 0.3-kb plasmid lacks these sites.

DISCUSSION

Genistein, which may play a major role in mediating the chemopreventive effects of soybeans, appears to act on multiple targets with the cell, including inhibition of PTK and DNA topoisomerase II activity.[3, 7] Among the various biological activities of genistein, anti-carcinogenic activity has attracted a great deal of attention. The basic mechanism through which genistein blocks cell cycle progression is now being elucidated. Recent experimental studies have shown that purified genistein induced G2/M arrest of a wide range of cancer cells, including leukemia, lymphoma, gastric, breast and prostate cancer, and melanoma.[14–19] In this regard, genistein differs from other flavones and even isoflavones such as daidzein, which induce G1 phase arrest.[48] However, the precise molecular mechanism(s) by which genistein perturbs cell cycle proliferation is still not clear. The purpose of the present report was to elucidate the molecular mechanism of action by which genistein inhibits the proliferation of cultured human prostate carcinoma PC-3-M cells. We have shown that genistein exhibits a strong reversible cell growth-inhibitory effect as determined by means of growth studies, cologenic analysis, and morphology (Figs. 1 and 2). Subsequent experiments addressed the issue of whether genistein perturbs the cell cycle by using DNA flow cytometric analysis. Flow cytometric data for genistein-treated cells clearly revealed a reversible cell-cycle block at the G2/M transition (Fig. 3) as expected from previous studies. We have also extensively investigated the possibility that genistein may cause apoptosis under our experimental conditions, because previous studies showed that genistein and other flavonoids can induce apoptosis in several cell lines.[14, 15, 18, 21] However, we could not detect any significant apoptotic cell death by several methods including DAPI staining, DNA fragmentation gel electrophoresis, flow cytometry, and TUNEL assays (data not shown). These results suggested the modulation of cell cycle-regulatory proteins and associated kinase activity as a possible molecular mechanism of the effect of genistein.

Cell cycle progression is regulated through several different Cdk regulatory mechanisms.[22, 23] Two major mechanisms for Cdk regulation are binding with its catalytic subunit cyclin, followed by activation of Cdk/cyclin complexes, and binding with Cdk inhibitors, followed by inactivation of Cdk/cyclin complexes. An alteration in the formation of these complexes could lead to an increased cell growth and proliferation, and decreased cell growth and proliferation followed by differentiation and/or cell death by apoptosis.[32, 33, 40] In mammalian cells, cyclin A is produced in late G1 phase and its expression accumulates during S and G2 phase, when it binds with and activates Cdk2.[28, 29] Cdc2, also called maturation promoting factor, acts as a serine/threonine kinase, that is activated by binding with cyclin B1 to initiate the M phase of the cell cycle.[30, 31] Increased kinase activity induces the phosphorylation of histone H1 and laminin of the nuclear membrane, followed by breakdown of the membrane and

chromosome formation, leading the cells to the start of mitosis.[49–51] Based on these reports, we next investigated the effects of genistein on the expression of G2/M regulatory proteins using western blot analysis. The results from the immunoblotting analyses demonstrated that genistein selectively down-regulates the intracellular protein levels of cyclin B1 (Fig. 4), which plays an essential role as a positive regulator of Cdc2 activity in cell cycle progression at the G2/M transition stage. We further examined whether genistein inhibits Cdks kinase activity using histone H1 as a substrate. The results from the immuno-complex kinase assays using specific antibodies to Cdk2 and Cdc2 demonstrated that genistein down-regulates both Cdk2 and Cdc2 kinase activities, rather than altering the protein levels, in a time-dependent manner (Fig. 4). These results were consistent with the effective times for significant induction of G2/M arrest of the cell cycle. Taken together, these results in part indicated that genistein suppresses cell proliferation by down-regulating the expression of cyclin B1, and the activities of Cdk2 and Cdc2 kinase.

The down-regulation of Cdk2 and Cdc2 kinase activities and the cell cycle arrest by genistein may also involve additional inhibitory mechanism(s). To test this hypothesis we next investigated whether Cdk inhibitors are involved in the genistein-induced growth arrest using western and northern blot analyses and co-immunoprecipitation. The hypothesis was clearly supported by the facts that genistein selectively induces expression of the protein and mRNA of Cdk inhibitor p21, and the increased p21 protein was tightly associated with Cdk2 and Cdc2 (Fig. 5). These results demonstrate that the down-regulation of Cdk2 kinase activity is mainly caused by selective induction of p21 expression, and the inhibition of Cdc2 kinase activity by genistein is due to both decreased expression of cyclin B1 and increased expression of p21 in human prostate carcinoma PC-3-M cells. p21 was first identified as a p53-inducible gene, which has further been shown to mediate the induction of cell cycle arrest at the G1 phase after DNA damage.[36–39] p21 can also be induced via a p53-independent mechanism in various cell lines stimulated for growth arrest and differentiation.[38, 40, 44, 45] Although p21 has been commonly associated with the G1 checkpoint, it has been shown that during the cell cycle of normal human fibroblasts, the levels of p21 mRNA peak twice, during G1 and G2,[46] which is suggestive of a potentional role of p21 in the G2/M phase. In addition, several studies have demonstrated that cell cycle arrest at the G2/M transition by DNA-damaging agents is also tightly associated with the induction of p21.[52, 53] This reciprocal down-regulation of cyclin B1 and induction of p21 and growth arrest, shown here in genistein-treated PC-3-M cells, is analogous to that seen in other breast carcinoma cell lines where cyclin B1 down-regulation also appears to be coupled to p21 induction and growth arrest.[19] Shao *et al.*[18] and Choi *et al.*[19] have recently shown that genistein can induce p21 expression in human breast carcinoma cells which have mutated *p53* genes. These results suggest that the induction of p21 by genistein may be independent of wild-type p53. Because the *p53* gene is truncated and p53 protein is absent in PC-3-M cells used in this study, these data demonstrate that genistein regulates the level of p21 expression through a p53-independent pathway. In addition, we observed that genistein activates the p21 promoter, and p53-binding sites are not required for the transcriptional activation by genistein. Using deletion constructs of the p21 promoter, we found that the response to genistein could be localized to the 300 base pairs proximal to the transcription start site. This region of the p21 promoter is GC-rich and contains a series of consensus binding sites for the Sp1 family.[36] Recent studies have shown that this region contains the binding sites for a number of agents that induce the p21 promoter, including transforming growth factor β, nerve growth factor, progesterone, lovastatin, butyrate and phorbol ester.[38, 45, 54–56] These results strongly suggest that Sp1 is involved in the transcriptional activation of the p21 promoter in response to genistein. To confirm this hypothesis, functional studies using mutant p21 promoter fragments are in progress.

In summary, the results obtained provide convincing evidence that genistein exerts its effect on cell cycle progression of PC-3-M cells via two pathways. First, by inducing a significant decrease in G2/M cyclin B1, and second, by inducing an increase in p21 expression that leads to increased binding with Cdc2 and Cdk2, resulting in a marked decrease of their kinase activities. When these results on cell cycle-regulatory proteins are compared with those obtained for cell cycle phase distribution and proliferation following genistein treatment of PC-3-M cells, it can be suggested that a p53-independent transcriptional regulation of p21 could be the major cause of the G2/M arrest effect and the antiproliferative action of genistein. These phenomena have not been previously described. Genistein and related isoflavonoid compounds may be promising candidates for cancer chemoprevention and treatment.

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