GENISTEIN-INDUCED G2/M ARREST IS ASSOCIATED WITH p53-INDEPENDENT INDUCTION OF Cdk INHIBITOR p21 *WAF1/CIP1* IN HUMAN CANCER 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) by 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 p21 promoter indicated that the response to genistein 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

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ABSTRACT

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) by 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 p21 promoter indicated that the response to genistein 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.

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INTRODUCTION

Several compounds in soybean have demonstrated anti-cancer activity. One of the hypothesized candidates against malignancy in soybean is genistein, the most abundant isoflavone presents in soybean and soy-based products, which was originally isolated from fermentation broth of *Pseudomonas* spp. (1). Akiyama *et al.* (1) were the first to describe the ability of genistein to specifically inhibit the protein tyrosine kinase activity. Other investigators have suggested that genistein may exert its effect through inhibition of DNA topoisomerase II and ribosomal S6 kinase activities, which may lead to protein-linked DNA strand breaks (2, 3). In addition, genistein has also been observed to act *in vitro* as a potent inhibitor of angiogenesis and development of metastases (4, 5). *In vivo* studies also demonstrated that genistein has to be effective in reducing tumourogenesis and carcinogenesis (6). Although a specific mechanism of action has not been identified, previous studies have showed that genistein inhibited the growth of wide range of cultured cancer cells, induced differentiation induction of several malignant cell lines, and caused apoptotic cell death (7-9). To date, despite these accumulated data, the molecular mechanism of its anti-proliferative action on cell cycle of cancer cells is poorly understood. In the present study, we examined the effect of genistein on the growth of human prostate carcinoma PC-3-M cells that lacks wild type p53.

MATERIALS AND METHODS

Cell culture, genistein treatments, growth study and morphology. PC-3-M cells were grown in RPMI 1640. Genistein was dissolved in dimethyl sulfoxide for all experiments. For growth inhibition analysis, cells were cultured in the presence or absence of genistein in culture medium. After 72 h of culture, the viable cells were scored using the Trypan Blue method. For

the morphological study, cells were grown on coverslips, treated with genistein and Wright-stained.

DNA flow cytometric analysis. Cells were fixed in 75% ethanol, washed with PBS and suspended in cold propidium iodide (PI) solution. Flow cytometry analyses were performed on a FACScan flow cytometry system.

Immunoprecipitation, Western immunoblotting and immuno-complex kinase assay. Cell extracts were incubated with immunoprecipitating antibody in extraction buffer. The immuno-complexes were precipitated with protein A-Sepharose beads. Western blot analysis was performed as described by Choi *et al.* (8). For *in vitro* kinase assay, cell lysates were incubated with ere hybridized with excess amount of [^{32}P]-labeled cDNA probes of p21, and theantibodies and precipitated with protein A-Sepharose. Kinase assay of immunoprecipitates was performed in reaction buffer containing histone H1 and [γ-^{32}P] ATP.

Northern blot analysis. 20 mg of total cellular RNA were denatured, electrophoresed on agarose-formaldehyde gel and blotted onto nylon hybridization membrane. After prehy-bridization, membranes were hybridized with excess amount of [^{32}P]-labeled cDNA probes of p21, and then washed under highly stringent conditions.

p21 promoter-luciferase constructs and transfection assay. Cells were transiently transfected with p21 promoter-luciferase reporter constructs using LipofectAMINE. Following transfection the cells were incubated for 12 h, and the cells were incubated for an additional 36 h in the presence or absence of genistein. The cells were then lysed, and luciferase activity was assayed using a Dynatech ML1000 luminometer.

RESULTS AND DISCUSSION

Genistein inhibits the cell proliferation and induces morphological change. On day 3, the growth of cells was inhibited to 58 or 77% of the control by genistein at 50 or 100 μM. Control cells had epitherial morphology and tended to grow in clusters with a somewhat acinar appearance. In contrast, cells developed features of neuronal morphology such as bipolar or multipolar cells with long processes and beaded varicosities after addition of genistein (data not shown).

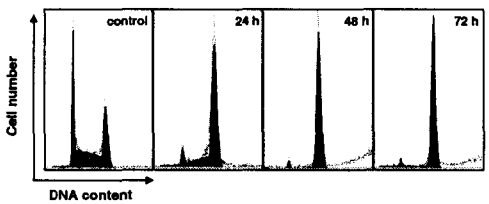
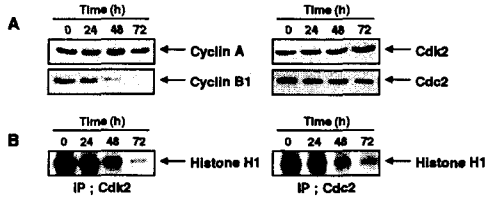


Fig. 1. DNA-fluorescence histogram. Exponentially growing cells at 60-70% confluency were treated at 0 time with 100 μM genistein. The cellular DNA was stained with PI, and analyzed by flow cytometry at different time points after genistein exposure.

Genistein induces G2/M arrest. The population of G1 and S cells continuously decreased in a time-dependent manner after exposure to genistein (Fig. 1). After 48 h treatment greater than 90% were arrested in G2/M, while very few G1 cells were observed.

Genistein inhibits expression of cyclin B1 and Cdks kinase activity. Since genistein treatment perturbed the G2/M phase of the cell cycle, we therefore determined the expression of intracellular proteins of cell cycle regulating components at the G2/M boundary in response to genistein treatment. Western blot analysis showed no significant change in the intracellular protein levels of cyclin A, Cdk2 and Cdc2 (Fig. 2A). 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 regulation of Cdc2 activity at G2/M phase. There it suggested that the genistein-induced growth arrest is partially due to its down-regulating effect on the intracellular levels of cyclin B1. We further determined whether genistein inhibits the Cdks kinase activity.



Both Cdc2 and Cdk2 kinase activities were significantly inhibited in a time-dependent manner in response to genistein treatment (Fig. 2B). This result also agreed well with respect to the effective times of genistein that induce cell cycle arrest and inhibit 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.

Fig. 2. (A) Total cell lysates were prepared, and equal amounts of protein lystaes electrophoretically separated by SDS-PAGE, transferred to nitrocellulose membranes. Western blots were detected with indicated antibodies, and ECL detection. (B) Total cell lystaes were prepared and immunoprecipitated with anti-Cdk2 or anti-Cdc2 antibody, and kinase activity was assayed using histone H1 as substrate.

Genistein induces p53-independent expression of p21 and asso-ciation of p21 with Cdks. Incubation of cells with genistein caused a striking time-dependent increase in the magnitude induction of p21 protein and mRNA between 24 to 48 h (Fig. 3). As p53 gene is deleted in PC-3-M cells, it is most likely that the induction of the p21 is mediated through a p53-independent fashion. Because it was well known that p21 inhibits the activity of Cdks by direct association with cyclin/Cdk complexes, so that the complex formation of cyclins/Cdks/ p21 is increased in cells arrested by DNA damaging agents (10). We therefore inves-tigated whether the observed decrease in Cdks kinase activity was due to an increase in their binding to the induced levels of p21 protein following genistein treatment. Association of p21 with Cdks was almost undetectable in control cells, however, treatment of cells with genistein resulted in a marked increase in the binding of Cdk2 and Cdc2 with p21. These results demonstrate that the down-regulation of Cdks kinase activity by genistein treatment is caused by highly induced p21 expression.

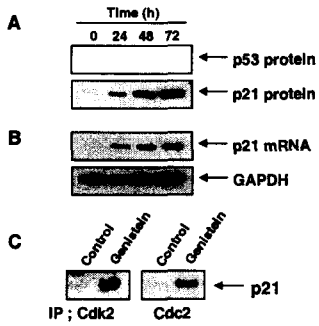
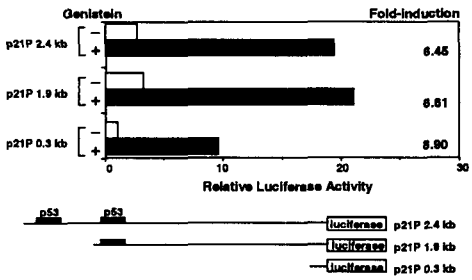


Fig. 3. Induction of p21 expression (A, Western blot analysis; B, Northern blot anlaysis) and association of p21 with Cdk2 and Cdc2 by genistein (C).

Genistein stimulates p21 promoter activity. We subsequently investigated whether the up-regulation of p21 expression by genistein involves the transcriptional regulation of the p21 gene promoter. Cells were transiently transfected with p21 promoter-luciferase fusion plasmids and luciferase activity was measured (Fig. 4). A 1.8-kb construct lacking a p53 response element was also 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 0.3-kb construct consisting of 300 base pairs most proximal to the transcription start site (11). This result suggests that the genistein-responsive site is localized to the promoter region within 300 base pair region relative to 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 0.3-kb plasmid lacks these sites.

In summary, the results obtained provide convincing evidence that genistein exerts its effect on cell cycle progression of PC-3-M cells by two pathways. First, by a significant decrease in G2/M cyclin B1, and second, by an increase in p21 expression that leads to its increased binding with Cdc2 and Cdk2, resulting in a marked decrease in their kinase activities. When these cell cycle-regulatory protein results are compared with those obtained for cell cycle phase distribution and proliferation following genistein treatment, it can be suggested that a p53-independent transcriptional regulation of p21 could be the major cause of the effect of genistein on G2/M arrest and on



anti-proliferative response.

Fig. 4. The p21 promoter constructs fused to the luciferase gene were transfected into cells, and the cells were incubated for 36 h in the presence of 100 μM genistein.

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