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American Ginseng Transcriptionally Activates *p21* mRNA in Breast Cancer Cell Lines

American ginseng (AG) has been demonstrated to inhibit breast cancer cell growth in vitro. p21 protein, a universal cell cycle inhibitor, binds cyclin-CDK complexes, an important mechanism in cell cycle regulation. The purpose of this investigation was to determine if AG induces p21 gene expression in hormone sensitive (MCF-7) and insensitive (MDA-MB-231) breast cancer cell lines. Cells grown in steroid stripped medium (SSM) were treated with AG, 17-β-estradiol (E_2), genistein or cycloheximide (CHX). Northern blot analyses were performed using human *p21*Cip1 and 36B4 cDNA probes. Cell lines were transiently transfected with select mouse *p21* CAT reporter constructs, including those lacking a p53 binding site. Cell cycle analyses was performed by FACScan. The results revealed that AG induced *p21* mRNA expression in MCF-7 and MDA-MB-231 cells (p=0.0004; $p \leq 0.0001$, respectively). Neither E_2 nor genistein alter *p21* mRNA expression. CHX, a protein synthesis inhibitor, did not block *p21* mRNA expression induced by AG, indicating that p21 is induced as an immediate early gene. AG activated *p21* reporter constructs in transfected cells, independent of p53 binding sites. The cell cycle proliferative phase was significantly decreased by AG and increased by E_2 ($p \leq 0.0001$). AG may inhibit breast cancer cell growth by transcriptional activation of the *p21* gene, independent of p53.

Key Words : *p21; American Ginseng; Breast Neoplasms*

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INTRODUCTION

Epidemiological studies have analyzed the impact of dietary components on the incidence of breast cancer (1-7). While there has been no clear consensus on the specific link between breast cancer development and diet, one study reported that soya products rich in phytoestrogens may protect against breast cancer in premenopausal women (1). Foods containing soybean and ginseng contain large quantities of the isoflavones genistein and daidzein, which are phytoestrogens with weak estrogen agonist activity that may interfere with the breast cancer promoting effects of physiologic estrogen (3). Increased soy protein consumption is significantly correlated with a reduction in the risk of sex steroid implicated cancers such as breast cancer and prostate cancer (1, 4). While a meta-analysis supported a reduced risk of breast cancer development with intake of vegetables and to a lesser extent fruit (6), a pooled analysis of cohort studies could verify no significant reduction in breast cancer risk with either fruit or vegetable consumption in the adult years (7).

The molecular basis of the effects of ginseng on cell proliferation has not been identified. Previous studies have shown that American ginseng induces the expression of *pS2*, an estrogen regulated gene (8). AG has also been shown to exert inhibitory growth effects on the hormone dependent MCF-7 breast cancer cell line (8, 9).

This present investigation focuses on determining a possible mechanism of action that may account for the breast cancer cell growth inhibitory properties of AG. The shift from quiescence (G_0) to an actively growing state is a prerequisite for entry into the cell cycle in most cells, and it is crucial step for cancer cells (10). Cell cycle progression is modulated by certain types of regulators known as cyclin kinase inhibitors. The first of these proteins to be identified and cloned was p21 (11-13). The p21 protein, a universal cell cycle inhibitor, binds cyclin-CDK complexes and proliferating cell nuclear antigen, thereby serving as a potent growth inhibitor and effector of cell-cycle checkpoint (14). Induction of p21 expression has been linked to growth inhibition by p53 (11), and also has been found to signal growth arrest, independent of p53 in cells undergoing differentiation (15-17).

The purpose of this investigation was to determine if American ginseng induces the cell cycle inhibitor p21 in hormone sensitive MCF-7 and insensitive MDA-MB-231 breast cancer cell lines.

METHODS

Cells and cell culture

MCF-7 (estrogen sensitive) and MDA-MB-231 (estrogen insensitive) breast cancer cell lines and the HT-29 colon cancer cell line were purchased from American Type Culture Collection (ATCC, Rockville, MD). The cells were grown in 75 cm^2 flasks for northern blot analyses, 100 mm culture plates for transient transfection and 25 cm^2 flasks for cell cycle analyses, respectively, at 37°C and 5% CO_2 in Eagle's Minimum Essential Medium (EMEM, Biowhittaker, MD) and supplemented with 10% fetal bovine serum, 584 mg/L L-glutamine and 5×10^{-2} mg/mL gentamicin until the cells were grown to 70% confluence. The medium was changed every 2 to 3 days and the cells were split by trypsinization when they reached confluence.

The breast cancer cell lines were stripped of endogenous steroid hormone by successive passages in phenol red free Dulbeco's Modified Eagle's Medium (DMEM, Biowhittaker, MD) containing 5% charcoal and dextran treated fetal bovine serum, 584 mg/mL L-glutamine, 0.2 IU/mL insulin and 5×10^{-2} mg/mL gentamicin (18).

After 7 days of steroid withdrawal, the breast cancer cell lines were treated with select concentrations (50 to 2,000 μg/mL) of a standardized American ginseng (AG) extract (*Panax quinquefolium* grown in Canada), (gift, CNT2000, Chi Na Ta Corp, Langley, British Columbia), 17-β-estradiol (E_2), (10^{-9} M, Sigma Chemical Company, St. Louis, MO), genistein (Gn), (10^{-6} M, Sigma) or cycloheximide (CHX), (10 μg/mL, Sigma) for 1 to 48 hr and the medium and test compounds were replenished at 24 hr (19).

HT-29 colon cancer cells were treated with 5 mM of sodium butyrate for four hours as the positive control group for *p21* mRNA expression (20).

Northern blot analyses

Total RNA was extracted by the single step method using Ultraspec RNA Isolation system (BIOTECX Laboratories, Houston, TX). Twenty micrograms of total RNA was electrophoresed onto a 1% agarose gel containing formaldehyde and transferred to a nylon membrane. The transferred RNA was crosslinked to a nylon membrane using UV stratalinker (Stratagene, La Jolla, CA). *p21*, a 1.009 kb Xho1/Ecor1 fragment derived from the human Cip1 cDNA (ATCC) and 36B4 cDNA were labeled with ^{32}P-dCTP by the random primer method (21) to a specific activity of 5×10^8 cpm/μg DNA using Oligolabelling kit (Pharmacia Biotech, Piscataway, NJ). The membranes were prehybridized for 16 hr, and then hybridized in 50% formamide/6× standard saline citrate (SSC)/0.5% sodium dodecyl sulfate (SDS)/5% Denhart's solution/100 μg/mL salmon sperm DNA/0.001 M EDTA at 42°C for 16 hr. The cells were washed twice in 2 × SSC/0.2% SDS, twice in 1 × SSC/0.2% SDS at room temperature and twice in 0.5 × SSC/0.2% SDS at 65°C. The washed filters were exposed to Kodak BioMax MR film at -70°C. The level of *p21* mRNA expression was determined by using laser densitometry and Image Quant software (Molecular Dynamics 400-B 2D, Sunnyvaly, CA) and normalized for RNA content using the *36B4* cDNA control.

Transient transfection and Chloramphenicol Acetyltransferase (CAT) Assays

The cells were grown at 50-60% confluence in 100 mm tissue culture plates and were transiently transfected with 10-20 μg of *p21* CAT reporter plasmids containing selected portions of the *p21* promoter constructs (gift, Tayler Jacks, Howard Hughes Medical Institute, Center for Cancer Research, MIT, Cambridge, MA.) from 0 to 4.7 kb upstream of the transcriptional start site or empty vector, *pJFCAT* by using the $CaPO_4$/DNA coprecipitation technique (Stratagene) (22). After 24 hr of transfection, the transfectants were treated with or without AG 2,000 μg/mL for 24 hr, then harvested by direct scraping followed by washing with phosphate buffered saline (PBS). Total protein was isolated in 150-200 μL of 0.25 M Tris-HCl. The concentration of the isolated protein was measured by CobasBio system (ROCHE Diagnostic, Nutley, NJ). Reaction products were applied onto a thin layer chromatography plate (23) and results of thin layer chromatography were recorded by standard instant photography using 55 positive negative film (Polaroid, Cambridge, MA) and were analyzed by densitometry scanning.

Flow cytometry

When the confluence of the cells reached 70% in 25 cm^2 flasks in standard or steroid stripped medium, the cells were treated with 10^{-9} M E_2 or 60 to 2,000 μg/mL AG. Culture media and test compounds were replenished at 24 hr. Forty-eight hours after either E_2 or AG treatment, cells were harvested by trypsinization, collected in 15 mL polyethylene tubes, and washed twice with phosphate buffered saline (PBS), and fixed in 70% ethanol. For DNA analyses, cells were centrifuged, and the pellet was stained with propidium iodide (PI, 50 μg/mL) dissolved in 50 μg/mL DNAse free RNAse and 0.1% Tritan X-100 (24-25). The cell pellets were dispersed by repeated pipetting and were placed at room temperature in the dark for 30 min. The DNA content fluorescence due to PI was measured by flow cytometry (FACScan, Becton Dickinson, San Jose, CA) on a minimum of 10,000 cells in the band above 620 nm. Data were recorded on Cell Quest software (Becton Dickinson) after an electronic gating of nuclear aggregates by the doublet discriminating module. The percentage of each cell cycle component was calculated from the histogram with ModFit (Becton Dickinson).

Statistical analyses

Fisher's PLSD of the ANOVA test and mean±s.d. were used for statistical analyses and a p-value less than 0.05 was considered statistically significant.

RESULTS

American ginseng induces *p21* mRNA

All experiments for northern blot analyses were performed in steroid stripped medium (SSM). Positive and negative controls, including cells grown in standard medium (SM) were utilized for all experiments. The doses of American ginseng (AG) tested ranged from 50 to 2,000 μg/mL and the time of treatment ranged from 1 to 48 hr.

AG induced a dose-dependent increase in *p21* mRNA expression in MCF-7 and MDA-MB-231 breast cancer cell lines. The greatest increase in p21 mRNA expression was identified with the AG 2,000 μg/mL treatment dose (p= 0.0004; $p \leq 0.0001$, respectively). Neither E_2 nor Gn induced *p21* mRNA expression in the MCF-7 or MDA-MB-231 breast cancer cell lines.

p21 mRNA induction by AG 2,000 μg/mL was identified as early as 1 hr after initiation of treatment in both MCF-7 and MDA-MB-231 cell lines and continued to be detected at an increased level at 48 hr of treatment. At 4 hr of treatment, AG induced a peak *p21* mRNA expression by approximately 4-fold in the MCF-7 cells and 3-fold in the MDA-MB-231 cells ($p \leq 0.0001$, both cell lines) in this time course experiment in comparison to the untreated SSM control group.

This increase in *p21* mRNA expression induced by the treatment of breast cancer cells by AG was equivalent to the increase in *p21* mRNA induced in the HT-29 colon cancer cell line positive control group treated with sodium butyrate. At 48 hr of treatment, there was no detection of *p21* mRNA induced by either E_2 or Gn in the MCF-7 or MDA-MB-231

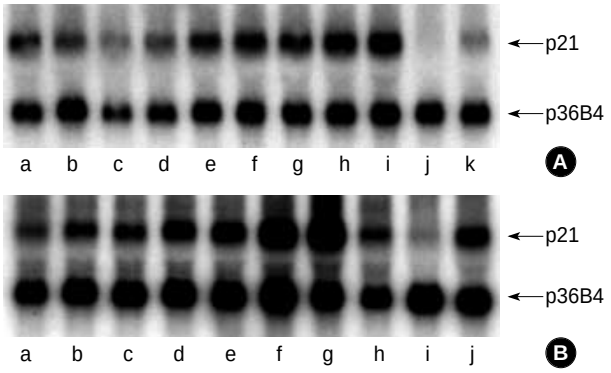

Fig. 1. Cycloheximide (CHX), the protein synthesis inhibitor, does not block *p21* mRNA expression by American ginseng.
Both MCF-7 (A) and MDA-MB-231 (B) breast cancer cell lines were treated with AG 2,000 μg/mL in the presence or absence of 10 μg/mL CHX for 4 hr and 24 hr. *p21* mRNA expression induced by AG was not blocked by concurrent treatment with CHX. There was no induction of *p21* mRNA by E_2 alone or in combination with CHX. There was a 3.8-fold increase in *p21* expression in MCF-7 cells treated with AG alone for 4 hr and a 4.2-fold increase in p21 expression when cells were treated with AG and CHX. At 24 hr of treatment, there was a 2.0-fold increase in *p21* mRNA expression induced when MCF-7 cells were treated with AG alone and a 14.8-fold increase when treated with AG and CHX compared to the untreated control group. A 2-fold increase in *p21* expression is noted for HT-29 cells treated with both sodium butyrate and CHX in comparison to sodium butyrate alone. In (A), the lanes correspond to the following treatments: a=SSM controls; b=E_2 (10^{-9} M) 4 hr; c=E_2 (10^{-9} M) 24 hr; d=AG 4 hr; e=AG 24 hr; f=E_2 and CHX 4 hr; g=E_2 and CHX 24 hr; h=AG and CHX 4 hr; i=AG and CHX 24 hr; j=HT-29 cells untreated control; k=HT-29 cells treated 4 hr with sodium butyrate; l=HT-29 cells treated 24 hr with sodium butyrate; m=HT-29 cells and sodium butyrate and CHX 4 hr; n=HT-29 cells and sodium butyrate and CHX 24 hr.
A 3.3, 1.2 and 1.5 fold increase in *p21* mRNA expression was detected for MDA-MB-231 cells treated with AG 2,000 μg/mL for 4, 24 and 48 hr, respectively, in comparison to untreated controls. There was a 4.7, 5.8, and 4.7 fold increase in *p21* expression when CHX was added to AG for the same time periods, respectively. In (B), the lanes correspond to the following treatments: a=SSM controls; b=AG 4 hr; c=AG and CHX 4 hr; d=AG 24 hr; e=AG and CXH 24 hr; f=AG 48 hr; g=AG and CHX 48 hr; h=CHX alone.

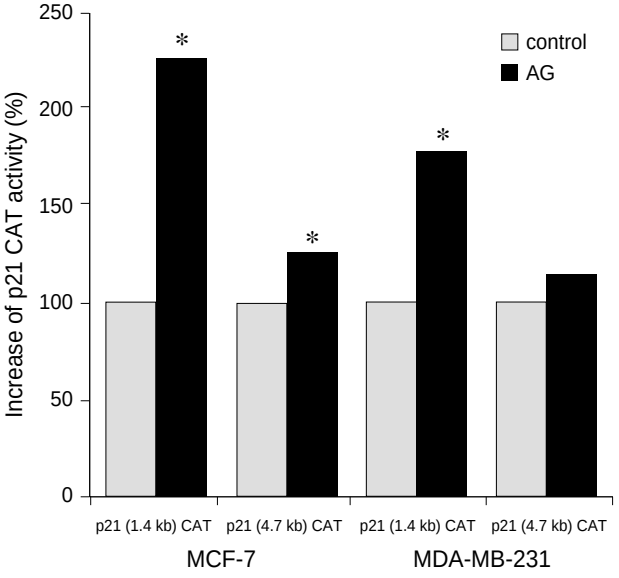

Fig. 2. American ginseng induces p21 promoter activity.
MCF-7 and MDA-MB-231 breast cancer cells were transiently transfected with 10 to 20 μg of murine p21 CAT reporter plasmid containing selected portions of p21 promoter constructs (from 0 to 4.7 kb upstream of the transcriptional start site). Transfectants were treated with AG 2,000 μg/mL for 24 hr. This histogram depicts the percent CAT induction of AG treated cells compared with the untreated negative control of p21 CAT activity in MCF-7 and MDA-MB-231 cell line, arbitrarily taken as 100%. *$p \leq 0.05$.

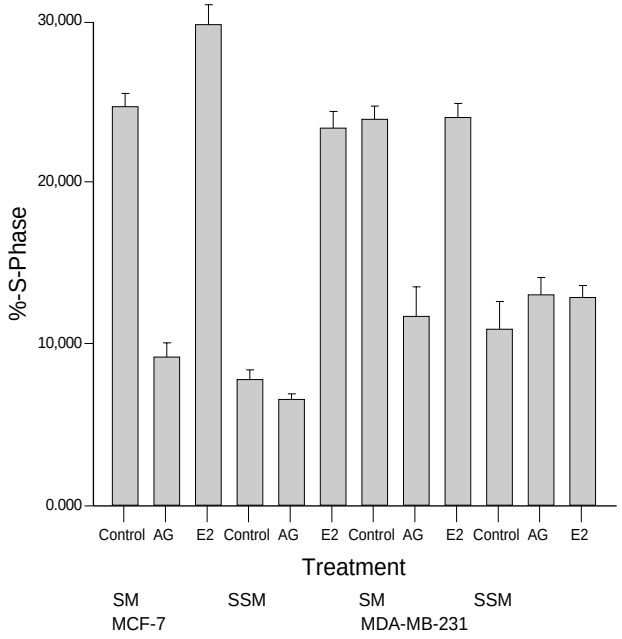

Fig. 3. Effect of American Ginseng and Estradiol on the Proliferative Phase (% S-Phase) of the Cell Cycle in Breast Cancer Cell Lines.
MCF-7 and MDA-MB-231 breast cancer cell lines were grown in either standard medium (SM) or steroid stripped medium (SSM) and treated for 48 hr with either AG (2,000 μg/mL), E_2 (10^{-9} M) or SM/SSM as a control group. DNA content was determined by FACScan flow cytometry (n=4). The graph represents the mean $\pm$s.d. For both cell lines grown in SM, AG significantly decreased the % S-phase ($p \leq 0.001$ for both) while AG had no significant effect on cells grown in SSM in comparison to the control groups. E_2 significantly increased the %-S-phase in MCF-7 cells grown in SM (p=0.022) and SSM ($p \leq 0.001$). E_2 had no significant effect on MDA-MB-231 breast cancer cells grown in either SM or SSM.

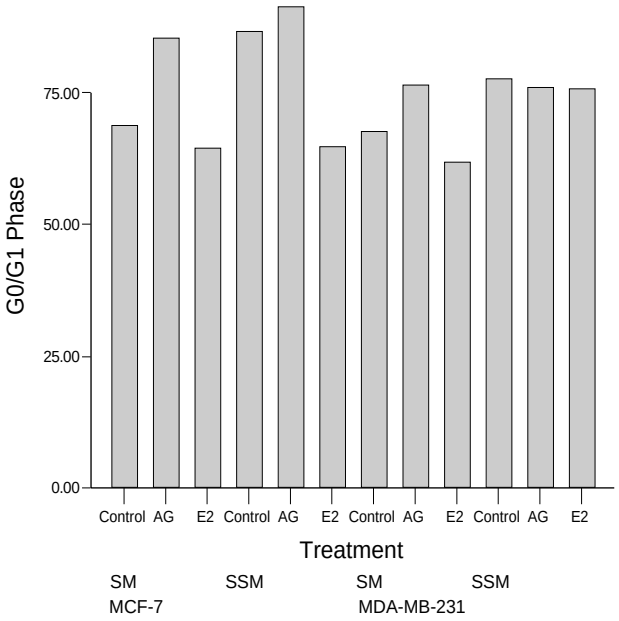

Fig. 4. Effect of American Ginseng and Estradiol on the Quiescent Phase (G0/G1) of the Cell Cycle in Breast Cancer Cell Lines.
MCF-7 and MDA-MB-231 breast cancer cell lines were grown in standard medium (SM) and steroid stripped medium (SSM) and treated for 48 hr with either AG (2,000 μg/mL), E_2 (10^{-9} M) or SM/SSM as a control group. DNA content was determined by FACScan flow cytometry (n=4). The graph represents the mean $\pm$standard deviation. For MCF-7 and MDA-MB-231 breast cancer cell lines grown in SM, AG significantly increased the G0/G1 phase ($p \leq 0.0001$ for both cell lines) in comparison to the respective control groups. AG also increased the G0/G1 phase in MCF-7 cells grown in SM ($p \leq 0.01$) but had no significant effect on MDA-MB-231 cells grown in SSM. E_2 had no significant effect on the G0/G1 phase of MCF-7 cells grown in SM or in MDA-MB-231 cells grown in SM or SSM. E_2 did significantly decrease the G0/G1 phase ($p \leq 0.0001$) in MCF-7 cells grown in SSM in comparison to the control group.

breast cancer cell lines.

Fig. 1A and B illustrates the results of the concomitant treatment of the MCF-7 and MDA-MB-231 breast cancer cell lines with AG and the protein synthesis inhibitor, CHX, respectively. This experiment demonstrated that *p21* mRNA expression induced by AG treatment is not blocked by CHX. Both AG and CHX increased p21 levels greater than either AG or CHX alone, indicating that p21 is induced as an immediate early gene and is superinducible by cycloheximide in wild type cells.

American ginseng induces *p21* gene expression in breast cancer cells, independent of p53 binding sites

The CAT reporter plasmids under the control of various portions of the mouse *p21* gene 5′ flanking region (from 0 to 4.7 kb upstream of the transcriptional initiation site) were transiently transfected into MCF-7 and MDA-MB-231 cells. The transfectants were treated with AG 2,000 μg/mL. AG induced p21 promoter activity in both MCF-7 and MDA-MB-231 cells, from 1.4 to 4.7 kb, even in those plasmids lacking the two p53 binding sites, located at -2.85 and -1.95 upstream from the transcriptional start site. AG activated the mouse p21 CAT plasmids in both cell lines, in comparison to that of the pJFCAT empty vector. The experiments were repeated four times to confirm the results. The results are shown in the histogram Fig. 2.

American ginseng induces growth arrest in breast cancer cells

Cell cycle analysis was performed in MCF-7 and MDA-MB-231 cell lines in steroid stripped medium (SSM) and standard medium (SM). It has been previously demonstrated that culture medium contains various growth factors and steroid hormones that may influence the outcome of hor-

monally related experiments (26), hence the effect on the proliferative (% S-phase) and resting phases (G_0/G_1) of the cell cycle was evaluated and compared in both mediums. Both cells lines were treated with either E_2 (10^{-9} M) or AG (2,000 μg/mL) and a negative control was used for all experiments.

In the MCF-7 cells grown in SM, E_2 significantly increased the % S-phase ($p=0.022$) and AG significantly decreased the % S-phase ($p \leq 0.001$) compared to the untreated control groups, as demonstrated in Fig. 3. E_2 had no effect on the G_0/G_1 phase while AG significantly increased this phase ($p \leq 0.0001$). For MCF-7 cells grown in SSM, E_2 significantly increased the % S-phase ($p \leq 0.001$) while AG exhibited a non-significant decrease of the proliferative phase in this medium ($p=0.10$). AG but not E_2 increased the G_0/G_1 phase ($p \leq 0.01$) in SSM cells.

The effect of AG on the % S-phase in MDA-MB-231 cells grown in SM was similar to that of the MCF-7 cells grown in SM. AG decreased ($p \leq 0.001$) the %-S-phase and increased the resting phase ($p \leq 0.0001$) while E_2 (p=NS) had no effect on either cell cycle phase in this estrogen insensitive cell line. Neither AG nor E_2 had any effect on the % S-phase or G_0/G_1 phase of MDA-MB-231 cells grown in SSM. The differences in apoptotic cell counts were minimal between controls and AG treated breast cancer cells (data not shown). Of note is the observation that the % S-phase for the control groups are significantly higher when the cell lines are grown in SM in comparison to SSM. Fig. 3 and 4 are histogram depictions of the results of the studies of the % S-phase and the G_0/G_1 cell cycle phases in MCF-7 and MDA-MB-231 cell lines.

DISCUSSION

The results from this study suggest that one possible mechanism of action of inhibition of breast cancer cell growth by AG may be through a molecular pathway involving the induction of the *p21* gene. Induction of *p21* by sodium butyrate in the HT-29 colon cancer cell line served as a model for this investigation. Butyrate induction of *p21* may represent a molecular link between a high fiber diet and the prevention of colon cancer (20).

One epidemiologic study reported that ginseng may contribute to a reduced risk of cancer development (27), but there is a paucity of data regarding the molecular mechanism involving the effects of ginseng on cell proliferation. In this present investigation, northern blot analyses were performed to examine the effects of American ginseng on the p21 expression. These studies established that AG induced *p21* mRNA expression in a time and dose dependent manner in both hormone sensitive (MCF-7) and insensitive (MDA-MB-231) breast cancer cell lines. Induction of *p21* mRNA expression was identified as early as 1 hr and peaked at 4 hr after AG treatment in MCF-7 and MDA-MB-231 cells. The protein synthesis inhibitor, cycloheximide (CHX) did not block American ginseng induced p21 expression, indicating that *p21* is an immediate early gene.

This rapid p21 induction is similar to that seen with many early genes such as *Egr-1* (28), *c-fos* (29), or *c-jun* (30) that are induced by mitogenic or other stimuli in the absence of de novo protein synthesis and thus constitute the first step in such a cascade. Macleod et al. showed that expression of the *p21* mRNA following serum stimulation is superinducible by cycloheximide in wild type primary mouse embryonic fibroblasts (22), similar to the results of these present studies.

Estrogenic steroids perform several major roles in mammalian physiology which include control of the development of the reproductive tract and secondary sex organs. They are also intimately linked with the development and progression of a number of human cancers, particularly breast cancer, by stimulating progression through G_1 phase of the cell cycle (31). Planas-Silva et al. (32) have shown that cyclin D1 has an important role in steroid-dependent cell proliferation and that estrogen, by stimulating the activities of G_1 cyclin-dependent kinases, can control the proliferation of breast cancer cells.

17-β-estradiol (E_2) was shown to exhibit no effect on *p21* mRNA expression in either estrogen sensitive or insensitive cell lines. E_2 increased the % S-phase fraction in MCF-7 cells but not in MDA-MB-231 cells. These cell cycle studies support previous studies that indicate that the MCF-7 breast cancer cell line is not only dependent on estrogen but that estradiol also has mitogenic effects in this estrogen dependent breast cancer cell line (19). This mitogenic property appears to be independent of p21 activation.

Several in vivo studies have revealed that estrogen and estrogen agonists, such as genistein and daidzein, can act as chemopreventive agents to inhibit the development of carcinogen induced mammary tumors (33-35). Hsieh et al. reported a biphasic proliferative effect of genistein on MCF-7 cells (36). This study suggested that low dose genistein (as low as 10^{-8} M) enhanced the proliferation of MCF-7 cells in vitro, with a concentration of 10^{-7} M achieving proliferative effects similar to those of 10^{-9} M estradiol. At concentrations above 2×10^{-5} M, however, genistein significantly inhibited MCF-7 cell growth. Since genistein had no effect on p21 expression, the mechanism of action for cell growth regulation must involve a different molecular event.

It has been shown that induction of *p21* following exposure to ionizing radiation or other DNA damaging agents requires p53 function (37). p53 mediates *p21* gene induction by transactivation through *cis*-elements located 1.95 and 2.85 kb upstream from the transcriptional initiation site in the mouse and 2.4 kb upstream in human *p21* gene (11, 22). p53 appears to be independent of p21 expression during normal mammalian development and cellular differentiation. This data from this present investigation suggests that AG may possibly achieve its growth inhibition in

breast cancer cells by transcriptional upregulation of cyclin dependent kinase inhibitor, p21, through one or more *cis*-element, in a p53 independent fashion.

Since AG induced *p21* mRNA expression in a time and dose dependent manner, flow cytometry studies were performed to determine whether AG would induce growth arrest in breast cancer cells. This data demonstrate that the % S-phase fraction of the cell cycle is significantly decreased by AG in the cells grown in standard medium and to a lesser degree in steroid stripped medium.

In the northern blot analyses, induction of *p21* mRNA expression by AG begins at a concentration of 50 μg/mL in MCF-7 cells and 500 μg/mL in MDA-MB-231 cells. The peak increase is found at 2,000 μg/mL in both MCF-7 and MDA-MB-231 cells. This is the same dose of this standardized AG extract that has been previously shown to induce mRNA expression of the estrogen regulated gene *pS2* to equal levels as E_2 10^{-9} M induced pS2 expression (38). In previous experiments, it had been shown that higher doses of E_2 did not further increase the levels of *pS2* mRNA expression in MCF-7 cells, hence this was the dose used in these current experiments. This increasing dose response of AG on *p21* mRNA expression is inversely related to the decrease of S-phase fraction of the cell cycle in standard medium, with the higher doses of AG resulting in a larger decrease in % S-phase. For both cell lines, the basal % S-phase fraction was higher in standard medium than steroid stripped medium. This is most likely secondary to the lack of growth factors and steroid hormones in SSM. Passage of cells with phenol red free medium containing 5% dextran charcoal treated fetal bovine serum followed by washing with phosphate buffered saline (PBS) removes not only endogenous steroids but also various growth factors (26).

Some investigators have shown that p21 binds tightly to the G_1 and S-phase kinases, cyclin E/Cdk2, cyclin D/Cdk 4, and cyclin A/Cdk2 and inhibits their activity, whereas p21 is a relatively poor inhibitor of the G_2/M phase kinase, Cyclin B/Cdc2 (39). G_0/G_1 phase was analyzed to investigate cyclin dependent kinase (CDK) inhibitor of cell cycle progression as a potential mechanism by which American ginseng negatively regulate cell proliferation. As shown in Fig. 4, AG induces G_0/G_1 arrest in both MCF-7 and MDA-MB-231 cells grown in standard medium, suggesting that the induction of the CDK inhibitor may be at least partly responsible for growth arrest induced by AG. Conversely, estradiol significantly decreases G_0/G_1 phase and increases % S-phase of the cell cycle in MCF-7 cells grown in standard medium, indicating a G_1/S transition and a mitogenic effect of E_2 on hormone dependent breast cancer cells.

The role of p21 in apoptosis is still not clear. Some investigators have shown that induction of apoptosis is associated with upregulation of endogenous p21 (40). However, others have reported that the *p21* gene is not essential for apoptosis (41) or protects the cells from apoptosis (42, 43). As determined by FACScan, this study found minimal differences in apoptotic cell counts between control and American ginseng treated cells. This result is consistent with the report of Hague et al., which showed minimal apoptosis in butyrate treated adherent colon cancer cells, but extensive apoptosis in those that were floating (44). Since adherent cells and not floating cells were harvested and subjected to FACScan in order to identify apoptosis, additional studies will be needed to determine if American ginseng induces apoptosis.

In conclusion, this investigation demonstrates that American ginseng exhibits breast cancer cell growth inhibitory properties by a mechanism that may involve transcriptional upregulation of cyclin dependent kinase inhibitor, p21, independent of p53.

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