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Role of cyclin inhibitor protein p21 in the inhibition of HCT116 human colon cancer cell proliferation by American ginseng (*Panax quinquefolius*) and its constituents

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Abstract

American ginseng and its ginsenoside constituents have been shown to exert anti-cancer effects although the mechanism of action remains unclear. The present study determined the effects of water-extracted ginseng (GE) or its ginsenoside (GF) and polysaccharide (PS) fractions on the proliferation of human colon cancer cells and examined the role of p21 in mediating these effects using wild-type and p21^{-/-} HCT116 human colon carcinoma cells. Proliferation was inhibited by GE, GF, and PS in wild-type and p21^{-/-} cells, and the p21^{-/-} cells were more sensitive to these treatments. Wild type cells treated with GE were arrested in the G0/G1 phase of the cell cycle and the expression of p53 and p21 proteins was increased while phospho-MEK levels decreased. In contrast, cells deficient in p21 displayed reduced cell viability, elevated number of dead cells, and increased expression of Bax and cleaved caspase-3 proteins. Both polysaccharides and ginsenosides appear to be responsible for the anti-proliferative and proapoptotic effects of GE. This study suggests that p21 functions to arrest HCT116 wild-type cells treated with GE, while p21-deficient cells undergo cell death in a ginseng constituent-dependent manner.

Keywords

American ginseng; HCT116 cells; colon cancer; p21; ginsenoside; polysaccharide

Introduction

The use of ginseng as an herbal dietary supplement has steadily risen in the United States (Barnes *et al.*, 2004), and has been a part of Traditional Chinese Medicine for centuries. Ginseng has been reported to have neuroprotective (Radad *et al.*, 2006), antidiabetic (Xie *et al.*, 2005), cardiovascular (Park *et al.*, 2005), and anti-cancer actions (Park *et al.*, 2005; Shin *et al.*, 2000; Kakizoe, 2000). Those studying its anti-cancer properties have focused largely on extracts of Asian (*Panax ginseng* CA Meyer) and American (*Panax quinquefolium*) ginseng and their biologically active constituents, the ginsenosides. Ginseng extracts have been shown to exert anti-proliferative and pro-apoptotic effects in a variety of cancer cell types (Helms, 2004; Hofseth and Wargovich, 2007; Kitts *et al.*, 2000), and the ginsenosides are believed to be the mediators of many of these actions (Helms, 2004; Kitts *et al.*, 2000; Oh *et al.*, 1999; Oh *et al.*, 2005; Kim *et al.*, 2004; Wang *et al.*, 2007a; Yang *et al.*, 2006).

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While studying the effects of individual ginsenosides has helped to identify potential mechanisms of ginseng action, it is also relevant to study whole root preparations similar to those that are commercially available for human consumption. Asian and American ginseng roots contain over 20 different ginsenosides as well as a large polysaccharide component, and polyacetylene alcohols. Ginseng polysaccharides have immunomodulatory activity and increase natural killer cell activity and cytokine release from immune cells *in vitro* and *in vivo* (Lim *et al.*, 2004; Shin *et al.*, 2002). The labile polyacetylenes have been shown to exert potent cytotoxic effects in lymphoma and gastric carcinoma cell lines *in vitro* (Kim *et al.*, 2002; Matsunaga *et al.*, 1994). In addition, several ginsenosides reportedly have anti-cancer effects, although it is unknown what interactions they may have with each other or other ginseng components in a whole root extract. Thus, the overall effect of ginseng may be quite different from that of any one component.

Recent work has identified the cyclin inhibitor protein p21 to be a potential key intermediary in the anti-cancer activity of ginseng and its ginsenoside components (Duda *et al.*, 2001; Liu *et al.*, 2000). Downstream from the p53 tumor suppressor, p21 plays a vital role in cell cycle arrest in response to DNA damage. As a member of the cyclin-dependent kinase (CDK) inhibitor protein family, p21 acts by binding cyclin-CDK complexes, inhibiting their kinase activity and effectively blocking DNA synthesis and cell cycle progression. Other studies have reported that ginseng extract and ginsenosides can induce cancer cell death through mechanisms involving the intrinsic (mitochondrial) apoptotic pathway, that include changes in protein expression of Bax, Bid, caspase-3, and PARP, as well as changes in mitochondrial membrane potential and cytochrome c release (Oh *et al.*, 2005; Yang *et al.*, 2006; Koo *et al.*, 2007; Oh and Lee, 2004).

The effects of ginseng extract on colon cancer cells have received little scientific investigation to date. Ginseng extract may exert a chemopreventative effect in the development of colon cancer (Wargovich, 2001; Volate *et al.*, 2005; Wang *et al.*, 2007c; Jin *et al.*, 2008) and has been shown to produce a synergistic inhibitory action on HCT116 human colon cancer cells when given in combination with 5-fluorouracil (Wang *et al.*, 2007b), as well as induce mitochondrial mediated apoptosis in SW-480 human colon cancer cells (Wang *et al.*, 2009). Using parental and an isogenic subline negative for *p21*, the goal of the present study was to determine the actions of American ginseng extract (GE) on HCT116 human colon cancer cells and identify the role of p21 and ginseng constituents in the ability of ginseng to inhibit HCT116 cell proliferation.

Materials and Methods

Water extraction of ginseng and compositional analysis

Certified pure American ginseng root powder (Ginseng Board of Wisconsin, Wausau, WI) was extracted in sterile deionized water as previously described (King and Murphy, 2007). The liquid extract was lyophilized and the resultant powdered ginseng extract (GE) stored at -20°C .

Ginsenoside content was determined as previously described (Wang *et al.*, 2007b,c). The HPLC system was a Waters 2960 instrument (Milford, MA) with a quaternary pump, automatic injector, a photodiode array detector (Model 996), and Waters Millennium software for peak identification and integration. The separation was carried out on a Prodigy ODS-2 column (5 μ , 3.2 $\times$ 250 mm I.D., Phenomenex; Torrance, CA) with a guard column (5 μ , 7.5 $\times$ 3.2 mm I.D., Phenomenex). 20 μ l of each sample were injected into the column and eluted at room temperature with a constant flow rate of 1.0 ml/min (Fig. 1). Acetonitrile (solvent A) and water (solvent B) were used in the mobile phase. Gradient elution proceeded as follows: started with 17.5% solvent A and 82.5% solvent B; changed to 21% A over 20 min; changed to 26% A

over 3 min; held for 19 min; changed to 36% A over 13 min; changed to 50% A over 9 min; changed to 68% A over 9 min; changed to 80% A over 2 min; held for 3 min; changed to 17.5% A over 5 min; held for 6 min. The detection wavelength was set to 202 nm.

Combined gas chromatography/mass spectrometry (GC/MS) analysis of the per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced by acidic methanolysis was used to determine glycosyl composition as previously described (Merkle and Poppe, 1994; York, *et al.*, 1985). Briefly, an aliquot of sample was added to separate tubes with 40ug and 60ug of Inositol as the internal standard. Methyl glycosides were then prepared from the dry sample by mild acid methanolysis with 1 M HCl in methanol at 80°C for 16 hours, followed by re-*N*-acetylation with pyridine and acetic anhydride in methanol. The sample was then per-*O*-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80°C for 30 minutes. GC/MS analysis of the TMS methyl glycosides was performed on an AT 6890N GC interfaced to a 5975B MSD, using a Supelco EC-1 fused silica capillary column (30 m × 0.25 mm ID).

Ginsenoside content of GE was 6.89% w/w (Fig. 1); carbohydrate content was 92% w/w, as determined by phenol-sulfuric acid reaction, and consisted of 99.3% glucose, 0.3% arabinose, 0.2% galactose, and 0.1% rhamnose; and protein content was 3.0% w/w, as determined by BCA assay.

Ginsenoside extraction

The ginsenoside fraction of American ginseng root was prepared by combining 10 g root powder with 190 ml methanol. This suspension was stirred for 24 hours at room temperature, centrifuged at 1200g for 15 min and supernatant was collected. Pelleted material was resuspended in a second equal volume of methanol and the extraction process was repeated. Final pellets were saved for further processing. The supernatants containing alcohol-soluble ginsenosides and other lipophilic components were combined and liquid was evaporated by vacuum centrifugation at 37°C, yielding approximately 1.5 g of product (15% w/w of starting material). The final powdered ginsenoside-rich fraction (GF) was stored at -20°C. Ginsenoside content of GF was 22.2% w/w (Fig. 1) and protein content was 1.96% w/w.

Ginseng polysaccharides extraction

Final pellets obtained from ginsenoside extraction described above were washed once with ethanol and polysaccharides were extracted as previously described (Assinewe *et al.*, 2002) with modifications. Briefly, pellets were resuspended in 190 ml sterile deionized water, and this solution was agitated for one hour at 90°C, cooled briefly at room temperature, and centrifuged at 1200g for 15 min. The supernatant was collected and pellets were resuspended in sterile deionized water at the original volume used and the extraction process was repeated. The aqueous supernatant from both rounds containing mostly polysaccharides was combined, frozen at -70°C, and lyophilized, yielding approximately 1g of product (10% w/w of starting material). The final powdered polysaccharide-rich fraction (PS) was stored at -20°C. Carbohydrate content was 51.8% w/w, consisting of 97.3% glucose, 1.5% galacturonic acid, 0.7% arabinose, 0.5% galactose, and 0.1% rhamnose; protein content was 6.5%; and ginsenoside content was <1%.

Cell Culture

HCT116 parental and *p21*^{-/-} cell lines (provided by Dr. Bert Vogelstein, Baltimore, MD) were maintained in McCoy's 5A medium (Sigma; St. Louis, MO) containing 10% FBS (Atlanta Biologicals; Lawrenceville, GA) and antibiotics (100 IU/ml penicillin G, 100 µg/ml streptomycin; Invitrogen; Carlsbad, CA). Cells were cultured at 37°C in a humidified atmosphere containing 95% air and 5% CO₂, and media was replaced every 2 days.

Cell proliferation assays

Cells were seeded in 24 well plates at a density of approximately 5×10^3 cells per well ($n=8$ wells per treatment group) and allowed to attach overnight. Extracts were prepared for *in vitro* use by dissolution of extract in desired amounts of cell culture medium overnight at 37°C followed by sterile syringe filtration (25mm PF 0.2 micron CA) prior to use. Cells were treated with a wide concentration range of test compounds (0–2.0 mg/ml ginseng extract, 0–0.2 mg/ml polysaccharide fraction, 0–0.5 mg/ml ginsenoside fraction) every 48 hours. Cells were trypsinized, collected, and counted using a Beckman Coulter automated particle counter after 48 hours or on day 6. Data from triplicate experiments were expressed as a percent of control (untreated) cells and plotted against log concentration. IC_{50} values were determined using linear regression.

Calcein assay for cell viability—Approximately 2×10^5 HCT116 cells were seeded in a 96 well plate and treated with or without 1.0 mg/ml ginseng extract, 0.1 mg/ml polysaccharide fraction, or 0.15 mg/ml ginsenoside fraction for 48 hours ($n=6$ wells/treatment group). Cells were washed with PBS and incubated with 2.5 μ M calcein-AM (Invitrogen) in PBS for 1 h at 37°C. Viable cells were determined by the ability of cell-permeable, non-fluorescent calcein-AM to be hydrolyzed to fluorescent calcein ion by intracellular esterases which was detected using a microplate reader with a fluorescein optical filter and excitation and emissions set at 485 and 528 nm, respectively. Data from triplicate experiments were expressed as percent of control (untreated) cells.

Trypan blue exclusion analysis of cell death

HCT116 cells were seeded in 24 well plates at a density of 1×10^5 cells per well and maintained as described above. Cells were treated for 48 hours with 1.0 mg/ml ginseng extract, 0.1 mg/ml polysaccharide fraction, or 0.15 mg/ml ginsenoside fraction ($n=8$ wells/treatment group). Trypan blue solution (Sigma) was added to each well for a final concentration of 0.1% and incubated for 5 min at 37°C. Trypan blue containing media was collected and saved from each well and attached cells were trypsinized. Immediately following trypsinization, trypan blue containing media was returned to each well and dead (blue) cells were counted manually using a hemacytometer. Data from triplicate experiments were expressed as percent of total cells in each group.

Flow cytometry for cell cycle analysis

Attached and floating HCT116 cells that had either been untreated (control) or treated with 1.0 mg/ml ginseng extract for 48 hours ($n=3$ samples/treatment group) were collected, washed with PBS containing 2% FBS, and centrifuged. Pellets were suspended in hypotonic buffer (0.5% Triton X-100 in PBS) containing RNase and incubated for 30 min at 37°C. Cells were then incubated with 50 μ g propidium iodide (Molecular Probes; Carlsbad, CA) on ice for 30 min. Fluorescent emission was quantified following excitation by FACScan cytometry (Beckton Dickinson; San Jose, CA) and analyzed using CellQuest software (Beckton Dickinson).

Western blot analysis

HCT116 cells were treated for 48 hours with 1.0 mg/ml ginseng extract, 0.1 mg/ml polysaccharide fraction, or 0.15 mg/ml ginsenoside fraction ($n=3$ samples/treatment group). Cells were lysed by incubation in ice cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 2.5 mM Na_2HPO_4 , 50 mM NaF) with protease and phosphatase inhibitors (Sigma). Lysates were centrifuged at 12000g for 10 min at 4°C and supernatants were collected. Protein concentration was determined by BCA assay (Pierce; Rockford, IL). Antibodies for p21, Bax, Bcl-2, MEK (Santa Cruz; Santa Cruz, CA), p53 and phospho-MEK1/2 (Cell Signaling;

Danvers, MA) were diluted for optimal signal. IR dye conjugated goat anti-mouse (Molecular Probes) and goat anti-rabbit (LiCor; Lincoln, NE) secondary antibodies were diluted 1:10000. The antigen-antibody complexes were detected using the Odyssey system (LiCor). Densitometry was performed using Odyssey software.

Cleaved Caspase-3 ELISA

Endogenous cleaved caspase-3 protein levels were detected using the PathScan® Cleaved Caspase-3 (Asp175) Sandwich Elisa Kit (Cell Signaling). Briefly, 100 µg of cell lysate from HCT116 cells treated with or without 1.0 mg/ml ginseng extract, 0.1 mg/ml polysaccharide fraction, or 0.15 mg/ml ginsenoside fraction (n=3 samples/treatment group) were transferred to supplied microplate wells coated with a total caspase-3 antibody. A biotinylated antibody for cleaved caspase-3 was then added to detect the captured protein, followed by HRP-linked streptavidin, and finally by HRP substrate for visualization. The optical density was read at 450 nm using a BioTek microplate reader. Data from triplicate experiments were expressed as fold change relative to control (untreated) cells.

Statistical Analysis

Values are reported as mean ± SEM. Analyses were performed using Stat View™ software and one-way ANOVA and Fisher's PLSD post hoc tests. A P value less than 0.05 was considered significant.

Results

Ginseng extract-induced inhibition of HCT116 human colon cancer cell proliferation is enhanced in cells lacking p21

Dose response studies were performed to determine the effects of water-extracted ginseng (GE) on wild type and *p21*-deficient HCT116 cell proliferation. A concentration dependent, inhibitory effect was seen when cells were exposed to a wide dose range (0–2.0 mg/ml) of GE for 6 days (Fig. 2A). The *p21*^{-/-} cells were more sensitive to the inhibitory effects of ginseng extract than their wild type counterparts, with an IC₅₀ of 0.46 ± 0.005 mg/ml compared to 0.79 ± 0.044 mg/ml, respectively. Cells were next treated for 48 hours with GE (1.0 mg/ml) to examine the effects of shorter exposure time (Fig. 2B). After just 48 hours of treatment, cell number was inhibited by 48.1% in *p21*^{-/-} cells and 36.8% in *p21*^{+/+} cells. Together, these results indicate that ginseng extract inhibits the proliferation of HCT116 human colon cancer cells, and cells deficient in *p21* appear more sensitive to this effect over time.

p21-dependent effects of ginseng extract on cell viability, cell death, and cell cycle arrest in HCT116 cells

To further study the anti-proliferative effects of ginseng extract on HCT116 cells with or without functional p21, cells were next treated with 1.0 mg/ml ginseng extract for 48 hours and examined for cell viability, cell death, and cell cycle distribution using calcein uptake, trypan blue exclusion, and flow cytometry, respectively. Although this concentration of ginseng extract produced a significant decrease (*p*<0.05) in HCT116 wild-type cell number (Fig. 2), GE had no effect on cell viability or cell death. However, in *p21*^{-/-} cells there was a 1.6 fold decrease in viable (Fig. 3A) and a 2.2 fold increase in dead cells (Fig. 3B). There was a slight but significant increase in the percentage of *p21*^{+/+} cells in the G0/G1 phase with a corresponding decrease in cells in the S phase following GE treatment (*p*<0.05), whereas *p21*^{-/-} cells exhibited significant accumulation (*p*<0.05) in the G2/M phase (Fig. 3C). These data suggest that the ability of ginseng extract to decrease the proliferation of HCT116 *p21*^{+/+} cells after 48 hours of treatment is primarily through a cytostatic mechanism, without compromised

viability. In contrast, HCT116 *p21*^{-/-} cells appear to arrest late in the cell cycle, leading to decreased viable cells as a result of cell death.

Ginseng extract alters the expression of proteins involved in cell cycle, cell signaling, and apoptosis

To examine the mechanism of ginseng extract-induced inhibition of HCT116 cell proliferation, the expression of several key proteins relevant to cell cycle, cell signaling, and apoptosis was analyzed by Western blot. As shown in Fig. 4A, wild type cells exhibited increased expression of the cell cycle regulatory proteins p53 and p21 after 48 hours exposure to 1.0 mg/ml ginseng extract, as well as decreased expression of the mitogen activated protein kinase signaling protein phospho-MEK. Expression of the pro-apoptotic protein Bax was also increased in a time dependent manner. In cells lacking *p21*, the only change in protein expression following treatment with GE was an increase in Bax levels. The changes in expression of Bax observed in both cell genotypes were not consistent with the observed cell death results (Fig. 2B). To clarify these data, the expression of the downstream mediator of apoptosis, cleaved-caspase 3, was analyzed by ELISA (Fig. 4B). In contrast to the expression of Bax protein, but consistent with changes in cell death, treatment of HCT116 *p21*^{+/+} cells with 1.0 mg/ml GE for 48 hours did not result in cleavage of caspase-3 protein, while levels of cleaved product were increased 2.6 fold in *p21*^{-/-} cells. Thus, HCT116 wild type cells with functional p21 undergo cell cycle arrest and have reduced cell signaling following ginseng extract treatment, whereas the predominant effect of ginseng extract in cells lacking *p21* is the induction of proteins involved in programmed cell death.

Polysaccharide and ginsenoside fractions of ginseng root differentially affect HCT116 cell proliferation, viability, and death in a p21 dependent manner

To begin to elucidate the components of ginseng root responsible for its anti-proliferative effects the major bioactive components of whole root, the ginsenosides and ginseng polysaccharides, were examined. Consistent with the inhibitory effects of ginseng extract (Fig. 2A), *p21*^{-/-} cells were more sensitive to ginseng polysaccharides (PS) ($IC_{50} = 0.09 \pm 0.006$ mg/ml) and ginsenosides (GF) ($IC_{50} = 0.1 \pm 0.001$ mg/ml) compared to wild type cells which exhibited IC_{50} concentrations of 0.2 ± 0.05 mg/ml and 0.18 ± 0.01 mg/ml, respectively (Fig. 5A). When cells were treated with concentrations equivalent to 1.0 mg/ml ginseng extract based on extraction yield (0.1 mg/ml PS or 0.15 mg/ml GF, see Materials and Methods) for 48 hours and analyzed for cell viability, neither fraction was able to alter the viability of *p21*^{+/+} cells while both polysaccharide and ginsenoside fractions decreased the number of viable *p21*^{-/-} cells by 29.4% and 26.1%, respectively (Fig. 5B, left). Cells were further analyzed for cell death, and although both fractions significantly inhibited the viability of *p21*^{-/-} cells, only treatment with ginsenoside fraction resulted in a 9.4% increase in the number of dead cells. In fact, GF was also able to induce cell death in wild type cells (Fig. 5B, right). These results suggest that both polysaccharide and ginsenoside fractions of ginseng root are responsible for the direct effects of ginseng extract on cell proliferation and viability after 48 hours of treatment, while the ginsenosides are capable of inducing cell death in both wild type and *p21*^{-/-}-HCT116 cells.

Ginsenosides, but not ginseng polysaccharides, induce Bax expression in HCT116 cells

To delineate the constituent contributions to the effects of ginseng extract on the pro-apoptotic proteins Bax and cleaved caspase-3, HCT116 cells were treated with 0.1 mg/ml polysaccharides (PS) or 0.15 mg/ml ginsenoside fraction (GF) for 48 hours and analyzed for Bax expression and cleaved caspase-3 levels. Wild type and *p21*^{-/-} cells displayed increased Bax expression after treatment with GF (Fig. 6). PS and GF were both able to increase cleavage of caspase-3 in *p21*^{-/-} cells by 2.3 and 2.2 fold respectively, although only treatment with

ginsenoside fraction caused a change in Bax expression. No changes in the expression of p53, p21, or phospho-MEK proteins were observed in either genotype after polysaccharide- or ginsenoside fraction treatment (data not shown). These results indicate that the ability of ginseng extract to stimulate Bax protein expression is mediated primarily by its ginsenoside fraction while both ginsenoside and polysaccharide fractions contribute to ginseng extract-induced caspase-3 cleavage.

Discussion

Colon cancer is the third most prevalent type of cancer and is the third leading cause of cancer-related deaths in the United States, with more than 100,000 new cases diagnosed each year (Jemal *et al.*, 2005). While ginseng has been reported to have potential chemopreventative effects relevant to colon cancer (Wargovich, 2001; Volate *et al.*, 2005; Wang *et al.*, 2007c; Jin *et al.*, 2008), little work has been done addressing its actions of its constituents on colon cancer cells. The metabolism, absorption, and bioavailability of pharmacological active constituents following oral ginseng intake have only recently begun to be addressed. The current study has shown that ginseng extract has a direct anti-proliferative effect on colon cancer cell proliferation *in vitro* and that both ginseng polysaccharide and ginsenoside fractions of whole root may contribute to this action. That cells deficient in the cell cycle inhibitor protein p21 displayed enhanced sensitivity to ginseng extract, polysaccharide fraction, and ginsenoside fraction, suggests that ginseng-induced activation of p21 in wild-type cells influences the inherent ability of the individual ginseng components to induce cell death. After oral administration, the ginsenosides can be processed by hydrolysis and conjugation to fatty acids, deglycosylation, or may pass through the intestine unchanged (Tawab *et al.*, 2003; Hasegawa, 2004; Bae *et al.*, 2004). Ginsenoside Rb1 is the most abundant ginsenoside in extracts (Corbit *et al.*, 2006a), and its intestinal metabolites, ginsenoside Rh2, compound K, and panaxydiol, have been shown to have potent anticancer activity *in vitro* (Helms, 2004). Studying of the effects of ginseng and its components on colon cancer is of particular importance considering the immediate availability of the active constituents, especially ginseng polysaccharides, in the intestine.

The proliferation of both *p21* positive and negative HCT116 human colon cancer cells was inhibited following treatment for 6 days with ginseng extract, and a significant anti-proliferative effect was seen as early as 48 hours after exposure (Fig. 2). Previous reports have shown that ginseng/ginsenosides increased expression of p21 in breast, prostate, and leukemia cell lines and reduced cell proliferation with apparent cell cycle arrest (Duda *et al.*, 2001; Liu *et al.*, 2000; Corbit *et al.*, 2006; King *et al.*, 2006). The current study also demonstrated decreased cell number (Fig. 2), cell cycle arrest in the G0/G1 phase (Fig. 3), and increased p53 and p21 expression (Fig. 4) after ginseng extract exposure in wild type HCT116 cells. Cell signaling was also inhibited, as evidenced by decreased levels of phosphorylated MEK. This finding is supported by our previous work with the MCF-7 breast cancer cell line where ginseng extract treatment decreased phosphorylated MEK and ERK1/2 levels, concomitant with increased Raf-1 kinase inhibitor protein (RKIP) expression (King *et al.*, 2007). Moreover, *p21*^{-/-} HCT116 cells were more sensitive to ginseng actions than their wild-type counterparts. Although *p21* activation has been reported to have both pro- and anti-apoptotic functions (Liu *et al.*, 2003; Gartel and Radhakrishnan, 2005), many studies demonstrate an anti-apoptotic function of *p21* in HCT116 cells. Loss of *p21* has been shown to increase chemotherapy- (Han *et al.*, 2002; Javelaud and Besancon, 2002; Le *et al.*, 2005; Lee *et al.*, 2006), hyperoxia- (Staversky *et al.*, 2006), and irradiation-induced apoptosis (Wendt *et al.*, 2006; Sohn *et al.*, 2006). Furthermore, the *p21*^{-/-} subline of HCT116 cells used in the current study has been shown to escape G2 arrest and enter aberrant mitosis following DNA damage, leading to morphological abnormalities, polyploidy and mitotic catastrophe (Bunz *et al.*, 1998). The

striking G2/M arrest seen here in *p21*^{-/-} cells treated with ginseng extract (Fig. 3) is consistent with these findings and suggests that these events likely precede cell death.

The ability of ginseng extract to induce the expression of the pro-apoptotic protein Bax was not influenced by *p21* status (Fig. 4). Bax is a member of the Bcl-2 family of proteins and, like *p21*, is regulated by *p53*. Bax functions to promote apoptosis by increasing mitochondrial membrane potential, allowing cytochrome c to be released. In the cytosol, cytochrome c complexes with the adapter protein Apaf-1 and activates the cysteine-protease caspase-9. Activation of the initiator caspase-9 leads to the caspase cascade, resulting in effector caspase-3 cleavage and activation followed by proteolysis throughout the cell and eventual death. In contrast to ginseng extract-induced Bax expression observed in both wild-type and *p21*^{-/-} cells, caspase 3 cleavage was only evident in *p21*^{-/-} cells (Fig. 4). It has been shown that *p21* activation may protect cells from irradiation-induced apoptosis downstream from the mitochondria by inhibiting CDK activity required for caspase-9 and subsequent caspase-3 activation (Wendt *et al.*, 2006; Sohn *et al.*, 2006), which could explain the results observed in the current study.

The inhibitory actions of ginseng extract on HCT116 human colon cancer cells prompted investigation into the role of the specific constituent fractions of the root. Interestingly, the abilities of polysaccharide and ginsenoside fractions to inhibit HCT116 cells were similar, but more potent than ginseng extract (Fig. 5A). These data suggest that both polysaccharide and ginsenoside fractions contribute to the ability of ginseng extract to inhibit HCT116 *p21*^{-/-} cell viability, while induction of cell death was primarily mediated by the ginsenosides (Fig. 5B). It was interesting that ginsenoside fraction was able to induce cell death in wild type cells while ginseng extract was without effect. Furthermore, while ginsenoside fraction was able to increase expression of pro-apoptotic proteins Bax and cleaved caspase-3, treatment with polysaccharide fraction resulted in elevation of cleaved caspase-3 levels without significant changes in Bax (Fig. 6). Taken together, these results suggest that the independent effects as well as interactions between ginsenosides and polysaccharides are involved in the overall net effect of ginseng extract. Further study is warranted using combinations of polysaccharide- and ginsenoside fractions, as well as individual ginsenosides and polysaccharides, to discern the nature of these interacting at the cellular level.

In conclusion, the current study demonstrates that American ginseng extract inhibits proliferation and cell cycle progression in HCT116 human colon cancer cells through altered expression of key regulatory proteins, and these actions appear to be dependent on the interaction of both ginsenoside and polysaccharide components of ginseng extract. Cells deficient for *p21*, however, undergo a cell death, likely involving mitochondrial mediated apoptosis, when treated with ginseng extract and this effect is influenced primarily by ginsenoside constituents. Activation of *p21* protein by the combined ginsenoside and ginseng polysaccharide components contained within the extract appears to prevent the pro-apoptotic actions of ginseng extract and its ginsenoside fraction. Although there is strong evidence that the ginseng polysaccharides exert anti-cancer activity through modulation of immune cells, we show for the first time a direct effect of ginseng polysaccharides on colon cancer cells *in vitro*.

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Fig. 1. HPLC analysis of ginsenoside content. Representative chromatograms are shown for (A) crude ginseng root extract (GE) and (B) ginsenoside rich fraction (GF). Ginsenoside concentrations shown in (C) were calculated using standard curves.

Fig. 2.

American ginseng root extract (GE) inhibits *p21*^{+/+} and *p21*^{-/-} HCT116 cell proliferation in a concentration and time dependent manner. **(A)** Cells were treated with GE (0–2.0 mg/ml) for six days and IC_{50} values were determined, or; **(B)** Cells were treated for 48 hours with 1.0 mg/ml GE and number of cells relative to control was determined. Data are displayed as a mean percent of untreated control $\pm$ standard error of the mean for three independent experiments.

* = $p < 0.05$ compared to controls.

Fig. 3.

The effects of 48 hours of treatment with 1.0 mg/ml ginseng extract (GE) on HCT116 cell viability, death, and cell cycle distribution are influenced by *p21* status. (A) Cell viability was determined by calcein uptake; (B) Dead cell number was determined by trypan blue exclusion; and, (C) Cell cycle distribution was determined by flow cytometry in *p21*^{+/+} (top panel) and *p21*^{-/-} HCT116 cells (bottom panel). Data are presented as a percent of untreated control or percent total cells $\pm$ standard error of the mean for three independent experiments. * = $p < 0.05$ compared to controls.

Fig. 4.

(A) Western blot analysis of cell cycle, cell signaling, and apoptosis related proteins were performed on *p21*^{+/+} and *p21*^{-/-} HCT116 cell lysates from cells treated for 24 or 48 hours with 1.0 mg/ml ginseng extract (GE). Actin was used as a loading control. Images shown are a representative from three independent trials. (B) Cleaved caspase-3 levels were determined by ELISA in lysates of cells treated with or without GE for 48 hours (n=3). Data are presented as GE-induced fold changes relative to untreated control. * = $p < 0.05$ compared to control.

Fig. 5.

The anti-proliferative effects of ginseng can be attributed to both polysaccharide (PS) and ginsenoside fractions (GF). **(A)** *p21*^{+/+} and *p21*^{-/-} HCT116 cells were treated with a range of PS (0–0.2 mg/ml, left panel) or GF (0–0.5 mg/ml, right panel) concentrations for six days and IC₅₀ values were determined, or **(B)** Cells were treated for 48 hours with 0.1 mg/ml PS or 0.15 mg/ml GF and analyzed for cell viability (left panel) and cell death (right panel). Data are displayed as the mean percent of untreated control or percent of total cells ± standard error of the mean for three independent experiments. * = *p* < 0.05 compared to controls.

Fig. 6. The expression of Bax protein (upper figures) and changes in cleaved caspase-3 (lower graph) in *p21*^{+/+} and *p21*^{-/-} HCT116 cells were analyzed in lysates obtained from cells treated with 0.1 mg/ml ginseng polysaccharides (PS) or 0.15 mg/ml ginsenosides (GF) for 48 hours (n=3). Actin was used as a loading control and representative Western blots are shown. Cleaved caspase-3 levels are presented as fold changes relative to untreated control (n=3). * = *p* < 0.05 compared to control.