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Combination of low dose of genistein and daidzein has synergistic preventive effects on isogenic human prostate cancer cells when compared with individual soy isoflavone

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ABSTRACT

The reduced incidence of prostate cancer (PCa) in Asia countries has been attributed to high soy diets, and major soy isoflavones, in particular daidzein and genistein, are thought to be the source of the beneficial and anti-cancer effects of soy foods. However, attention has been drawn to the safety of using high levels of soy isoflavones in humans, which is especially the concern for consumers taking regular soy isoflavone dietary supplements. The main objective of this study is thus to identify a soy isoflavone combination with lower levels of daidzein and genistein to be a more efficacious and safer chemo-preventive agent for PCa. The anticancer effects of daidzein and genistein, and their combinations on early-stage androgen-dependent PCa cells (LNCaP) and bone metastatic LNCaP-derivative PCa cells (C4-2B) were compared. Cells were treated with varying concentrations of daidzein, genistein (25–200 μM) or their combinations (25 or 50 μM) and cell proliferation, apoptosis, cell cycles and cellular uptakes of the isoflavones were measured after 48 h. Daidzein and genistein showed a synergistic effect on inhibiting cell proliferation and inducing apoptosis of both PCa cells. Twenty-five μM daidzein/50 μM genistein and 50 μM daidzein/50 μM genistein significantly increased the apoptotic effects on C4-2B cells although they did not show any effect when used individually. Except 50 μM daidzein/50 μM genistein, all other combinations had no impacts on cell cycles. For treatment with soy isoflavone combination, genistein was always better taken up than daidzein by both LNCaP and C4-2B cells.

1. Introduction

Prostate cancer (PCa) is the most common malignancy and second leading cause of cancer death in American men. The estimated new cases of PCa in United States were over one fifth million, and the deaths were over thirty thousand in year 2010 (NCI, 2011). The current treatments of PCa include surgery, radiation therapy and hormone therapy; however, unsatisfactory outcomes are associated. Besides, there is no curative and effective long-term treatment for bone metastatic PCa. Therefore, to develop novel preventive approaches to control this disease and slow the progression of the disease is necessary and urgent. Chemoprevention using natural dietary substance is one of the approaches that has been widely studied recently (Syed, Khan, Afaq, & Mukhtar, 2007). It is suggested that the lower incidence of PCa in Asian men compared to men in western countries might be attributable to dietary differences. The typical Asian diet is more soy-rich and increased soy consumption has been associated with a lower risk of prostate cancer (Yan & Spitznagel, 2005; Dalais et al., 2004). However, the exact mechanism is still unknown.

Soy isoflavones, such as daidzein and genistein, are believed to have an important role in reducing the incidence of prostate cancer. High doses of daidzein and genistein (>100 μmol/L) induced apoptosis and cell cycle arrest in benign prostate hyperplasia (BPH-1) cells, malignant androgen-independent prostate cancer epithelial cells (PC3) and early-stage androgen-dependent prostate cancer cells (LNCaP) (Hsu, Bray, Helferich, Doerge, & Ho, 2010). *In vivo* studies showed that dietary genistein reduced the incidence of advanced prostate cancer induced by *N*-methylnitrosourea in male Lobund-Wistar rats during adult and life-time exposure (Wang, Eltoum, Carpenter, & Lamartiniere, 2009). Recent studies have revealed that the inhibition of human prostate cancer cells induced by daidzein and genistein are related to the modulation of genes that are related to the control of cell cycle and apoptosis (Rabiau et al., 2010). Interestingly, soy extract containing mixture of daidzein and genistein and other compounds induced more apoptosis on PCa cells (PC-3 and LNCaP cells) as compared to purified

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daidzein or genistein. In addition, soy extract did not induce significant apoptosis in non-cancerous BPH-1 cells while both daidzein and genistein induced apoptosis in BPH-1 cells, suggesting that individual isoflavones may have higher cytotoxicity in non-cancerous cells (Hsu et al., 2010).

Nevertheless, attention has been drawn to the safety of using high levels of soy isoflavones in humans and the limited effect of individual soy isoflavones (Perabo et al., 2008). Both *in vitro* and *in vivo* experiments have shown controversial evidence as to whether these ingredients of soy, such as genistein, promote or inhibit tumor growth (Messina & Loprinzi, 2001). Due to reports on genistein immunosuppressive properties and negative impacts on thymic function (Divi, Change, & Dorge, 1997), concerns have been raised about the overconsumption of this soy isoflavone, especially among consumers taking regular soy isoflavone dietary supplements. To overcome the negative impacts from high doses of individual soy isoflavone, a soy isoflavone combination with lower levels of daidzein and genistein might be a more efficacious and safer chemo-preventive agent for PCa, because interactions between various bioactive phytochemicals may work synergistically to provide additional benefits. Zhou, Yu, Zhong, and Blackburn (2003) verified the synergistic inhibitory effect of soy phytochemicals and tea bioactive components on androgen-sensitive human prostate tumors in mice. Kim, Jeong, and Kim (2008) showed that soy extract is more potent than genistein in inhibition of tumor growth. However, the interactions may also be negative and undesirable (Lila & Raskin, 2005) and require careful evaluation.

Although lots of work has been done on the effects of these isoflavones on PCa, limited information is available about their effects on metastasis of PCa. The LNCaP progression model consisting of LNCaP, C4, C4-2 and C4-2B represents four stages of PCa progression (Thalmann et al., 2000). LNCaP is poorly metastatic and androgen sensitive, C4 is highly tumorigenic but poorly metastatic, C4-2 is castrate independent, aggressively tumorigenic and metastatic while C4-2B is castrate resistant and bone adapted. The LNCaP progression model shares remarkable similarities with human PCa (Thalmann et al., 2000), thus it can be an excellent model to study the potential activities of soy foods during PCa progression. In this study, the effects of daidzein, genistein and the combined effects of daidzein and genistein on the proliferation and apoptosis of early-stage androgen-dependent prostate cancer cells (LNCaP) and bone metastatic LNCaP-derivative prostate cancer cells (C4-2B) were evaluated. In addition, cell cycles and cellular uptakes of the isoflavones were measured after 48 h treatments.

2. Materials and methods

2.1. Materials

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), daidzein, genistein, docetaxel, resazurin, trifluoroacetic acid, acetonitrile, Triton X-100, propidium iodide and RNase A were obtained from Sigma–Aldrich (Saint Louis, MO, USA). Daidzein, genistein and docetaxel were made up as stock solutions in DMSO and stored at −20 °C. On the day of the experiments they were diluted with DMSO.

2.2. Cell culture

LNCaP and C4-2B cells were maintained in T-medium supplemented with 5% (v/v) fetal bovine serum (FBS) and 100 U/ml penicillin G sodium and 100 mg/ml streptomycin sulfate in 0.085% (w/v) saline (PS) in 5.0% CO_2 atmosphere at 37 °C. Medium was changed every 2 days. Cells were routinely passaged using 0.25% (w/v) trypsin with ethylenediaminetetraacetic acid (EDTA) 4 Na when 90–95% confluence was reached.

2.3. Treatment of cells

Cells were seeded in 24-well plates (Corning, Lowell, MA, USA) at a density of 1×10^5 cells per well. Cells were grown to 50–70% confluence and then treated with daidzein (25–200 μM), genistein (25–200 μM) or their combinations (25 or 50 μM of each, four combinations in total). The final concentration of DMSO used was 0.1% (v/v) for each treatment. Control cells treated with 0.1% DMSO served as the vehicle group. Fifty nM docetaxel was used as a positive control. After 48 h of treatment, cells were harvested and pelleted by centrifugation at 200*g* for 10 min. Pelleted cells were used for apoptosis assay immediately and the supernatant was kept at 4 °C for measurement of cytotoxicity.

2.4. Apoptosis assay

DNA fragmentation was measured using the Cell Death Detection ELISA^{PLUS} kit (Roche Applied Science, Indianapolis, IN, USA) following the protocol of the manufacturer. Cells were lysed with 200 μl lysis buffer and incubated at room temperature for 30 min. The lysate was then centrifuged at 200*g* for 10 min. Twenty μl supernatant was transferred into the streptavidin coated microplate and 80 μl immunoreagent was added to each well. The plate was covered with an adhesive foil cover and incubated on a shaker at 300 rpm for 2 h at room temperature. The solution was thoroughly removed and each well was washed three times with 300 μl incubation buffer. One hundred μl ABTS solution was added to each plate and the plate was incubated on a shaker at 250 rpm. When the color development was sufficient for a photometric analysis, 100 μl ABTS stop solution was added to each well. Colorimetric detection was carried out according to manufacturer's instructions using MRX microplate reader (Dynex Technologies, Chantilly, VA, USA) at 405 and 490 nm.

2.5. Cytotoxicity analysis

Cytotoxicity to cells was determined by measuring the amount of lactate dehydrogenase (LDH) enzyme leaked from the cytosol of damaged cells into the medium after exposure of cells to the chemicals for 48 h. The LDH release represents necrosis as opposed to apoptosis. LDH in the supernatant was measured using the Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche Applied Science, Indianapolis, IN, USA) following the protocol of the manufacturer. A total of 50 μl of the supernatant from the cells was placed in a 96-well plate, and 100 μl of LDH assay solution (mixture of catalyst lyophilizate and dye solution) was added to each well and incubated for 5 min in the dark at room temperature. Absorbance of the mixture was read with MRX microplate reader at 490 nm.

2.6. Measurement of proliferation

Antiproliferative activity was measured by a resazurin assay. Cells were seeded in a 96-well plate (Corning, Lowell, MA, USA) at a density of 1×10^4 cells per well. Cells were grown to 50–70% confluence and then treated with chemicals prepared in DMSO. The final concentration of DMSO used was 0.1% (v/v) for each treatment. Twenty μl 1 mg/ml resazurin solution (10% of medium, v/v) was then added into each well. After 48 h of treatment, the absorbance was measured at 570 and 600 nm using a Synergy™ 2 multimode microplate reader (BioTek Instruments, Inc, Winooski, VT, USA). Data was processed according to the following equation provided by the manufacturer: $\frac{117216\times A_{570}-80586\times A_{600}}{117216\times A^{\circ}_{570}-80586\times A^{\circ}_{600}}$ (A_{570} and A_{600}