Soy milk digestion extract inhibits progression of prostate cancer cell growth via regulation of prostate cancer-specific antigen and cell cycle-regulatory genes in human LNCaP cancer cells

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Abstract. Soy milk, which is produced from whole soybeans, contains a variety of biologically active components. Isoflavones are a class of soy-derived phytoestrogens with beneficial effects, among which genistein (GEN) has been previously indicated to reduce the risk of prostate cancer. The present study evaluated the effects of soy milk digestion extract (SMD) on the progression of prostate cancer via the estrogen receptor (ER)β in human LNCaP prostate cancer cells. To evaluate the effects of SMD (daizein, 1.988 mg/100g, glycitein, 23.537 mg/100 g and GEN, 0.685 mg/100g) on cell proliferation, LNCaP cells were cultured in media containing vehicle (0.1% dimethyl sulfoxide), 17β-estradiol (E2; $2.7x10^{-7}$ mg/ml), GEN ($2.7x10^{-2}$ mg/ml) of SMD (total aglycon concentration, 0.79 mg/ml), after which the cell viability was examined using an MTT assay. The cell viability was significantly elevated by E2 (by 45±0.18%), while it was markedly reduced by GEN (73.2±0.03%) or SMD (74.8±0.09%). Semi-quantitative reverse transcription polymerase chain reaction analysis was performed to assess the mRNA expression levels of target genes, including ERβ, prostate cancer-specific antigen (PSA) and cell cycle regulators p21, Cyclin D1 and cyclin-dependent kinase (CDK)4. The expression of ERβ was almost completely diminished by E2, whereas it was significantly elevated by SMD. In addition, the expression levels of PSA were considerably reduced by SMD. The expression of p21 was significantly elevated by SMD, while it was markedly reduced by E2. Of note, the expression levels of Cyclin D1 and CDK4 were considerably elevated by E2, while being significantly reduced by GEN and SMD. All of these results indicated that SMD may inhibit the proliferation of human prostate cancer cells via regulating the expression of ERβ, PSA, p21, Cyclin D1 and CDK4 in an ER-dependent manner.

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Introduction

Prostate cancer is the most common malignancy in males in Europe and North America, while being the second leading cause of cancer-associated mortality in American men (1,2) as well as the fifth most common cancer type in Korean men (3). Prostate cancer at the early stage requires androgen for survival and proliferation. Androgen activates androgen receptor (AR), which has an important role in the development, maturation and function of the prostate gland as well as the progression of prostate cancer (4). Thus, prostate cancer is initially treated by hormone manipulation, specifically androgen-ablative and anti-androgen therapy, to inhibit the development of metastasis (5). However, these treatments bear considerable cardiovascular side effects and most prostate cancers progress rapidly to become hormone resistant (6). Therefore, novel therapies and preventative methods are required to treat prostate cancer.

The mortality rate for prostate cancer in Asian men is lower than that in men residing in Western countries. Several studies have shown that the high consumption of soy products such as whole soybeans, soy milk and bean curd (tofu) in Asian countries may be associated with reduced risk of prostate cancer (7-10). In support of this, dietary habits have been shown to be associated with reduced risk of cancer (11-16). Soybeans, an important component of traditional Asian diets, is a rich source of isoflavones, saponin and plant protein (17). Soy milk, which is a product of hulled soybeans, contains a variety of

biologically active substances and has become a popular substitute for dairy milk (18). Soy milk may have beneficial effects on bone health and may reduce obesity due to the presence of isoflavones, saponin, calcium, vitamins and phytosterols (18,19). Of note, soy milk consumption (more than once a day) has been shown to reduce the risk of prostate cancer in Californian men by 70% (20). Genistein (GEN), a major isoflavone constituent of soybeans and soy products, has been reported to inhibit the growth of androgen-dependent as well as androgen-independent human prostate cancer cells (7,10). Isoflavones are phytoestrogens with beneficial effects, the most abundant of which are daidzein and GEN in soybeans, which also contain glycitein (21). Soy isoflavones are typically present in their glucoside form, which is not bioactive. After ingestion of soybeans or soy milk, isoflavone glucosides are hydrolyzed by intestinal mucosal as well as bacterial β-glucosidases into aglycones, which are absorbed more rapidly and in higher amounts (22,23). The overall bioavailability of the active components of soy is determined by gastrointestinal digestion, absorption and metabolism (24). The isoflavones in soy milk, including daidzein, genistein and glycitein, structurally resemble 17β-estradiol (E2) (25) and display differential binding affinities to the two estrogen receptors (ERs), ERα and ERβ (26,27). In particular, soy isoflavones bind to ERβ 20- to 30-fold more strongly than E2 (7). ERβ is highly expressed in LNCaP prostate cancer cells, whereas ERα expression is relatively low or undetectable (6). According to several studies, ERβ may have a role in the protection against malignant cell proliferation and metastasis through mediation of estrogen activity in prostate cancer (8,28).

Based on these previous studies, the present study hypothesized that intake of soy milk may have inhibitory effects on the growth of human prostate cancer cells, an effect exerted by combined isoflavones in soy milk biologically activated during digestion. To prove this hypothesis, a soy milk digestion extract (SMD) was produced by simulated gastrointestinal digestion *in vitro* to obtain aglycone isoflavones. The effects of SMD, GEN and E2 with or without inhibitors of ER or AD on the proliferative rate of LNCaP prostate cancer cells were assessed. Furthermore, the effects of these treatments on the expression of ERβ, AR, prostate cancer-specific antigen (PSA) and cell cycle regulatory genes were assessed. The present *in vitro* study suggested that ingestion of soy milk may prevent the development or progression of prostate cancer in an ERβ-dependent manner.

Materials and methods

In vitro digestion and isoflavone extraction. SMD was prepared in a manner similar to human gastrointestinal digestion followed by extraction of aglycone isoflavones, as previously described by Tate *et al* (29) with certain modifications. Soy milk (300 ml) was obtained from Chung's Food (Cheongju, Chungcheongbuk, Republic of Korea) and digested *in vitro*. The pH of the soy milk was adjusted to 2.8 with 6 N HCl (Merck kGaA, Darmstadt, Germany), followed by addition of 15 ml of a pepsin (Sigma-Aldrich, St. Louis, MO, USA) suspension (4 g pepsin/100 ml 0.1N HCl; Merck kGaA). The mixture was incubated with shaking at 37˚C for 2 h, after which the pH was adjusted to 5.7 with 5 M NaOH (Showa Denko, Tokyo, Japan). Subsequently, 75 ml pancreatin-bile salt mixture (0.2 g pancreatin and 1.2 g bile salts suspended in 100 ml 0.1 M $NaHCO_3$ (Sigma-Aldrich) was added to the mixture, followed by incubation as above for an additional 2 h. Following centrifugation at 1,096 x g for 20 min at room temperature, the solvent of the supernatant was concentrated using a rotary vacuum evaporator (BÜCHI Laboratechnik AG, Flawil, Switzerland) and freeze-dried using a lyophilizer (FD5512, Ilshin Lab Co., Ltd., Dongducheon-si, Korea).

The freeze-dried supernatant was extracted to determine the isoflavone concentration. The powder (0.2 g) was extracted with 80% methanol (Merck kGaA) at 65˚C in a water bath with shaking (130 rpm) for 2 h. After cooling to room temperature, 3 ml of 2M NaOH (Showa Denko) was added followed by shaking for 10 min (300 rpm). Extracts were decanted into a 50-ml tube and centrifuged at 3,500 rpm for 20 min at room temperature. The supernatant was then concentrated using a rotary vacuum evaporator (BÜCHI Labortechnik AG) and freeze-dried using a lyophilizer (Ilshin Lab Co., Ltd.). The yield was ~3.34%.

Isoflavone analysis by high-performance liquid chromatography (HPLC). The isoflavone concentration of the SMD obtained was analyzed by HPLC with ultraviolet detection (Agilent 1260 infinity; Agilent Technologies, Palo Alto, CA, USA). SMD powder was extracted in the same manner as above. Centrifuged supernatant was decanted and diluted into a 10-ml volumetric flask containing 4 ml ultrapure water (18.2 MΩ·cm) for HPLC analysis. After filtration using a 0.45-μm filter (17 mm nylon syringe filter; Thermo Fisher Scientific, Inc., Waltham, MA, USA) attached to a nylon syringe, extracts were filled into HPLC tubes. A Spherex5 C18 column (150x4.6 mm inner diameter; 5 μm; Phenomenex Co, Torrance, CA, USA) was maintained at a temperature of 40˚C. The mobile phase was composed of solution A [100% methanol, acetic acid and ultrapure water (44:5:1, v:v)] and solution B [100% methanol and acetic acid (49:1, v:v)], and a constant A/B ratio of 9:1 was used. Chromatographic separation was monitored at a flow rate of 1.5 ml/min. The concentrations of daidzein, glycitein, and GEN were measured at 260 nm. The standard material of 12 types of isoflavone isomer was used for isoflavone analysis. The stock solution contained a mixture of 12 types of isomers. The stock solution mixture was as follows: 0.2 mg/ml daidzin, 0.1 mg/ml glycitin, 0.2 mg/ml genistin, 0.2 mg/ml daidzein, 0.1 mg/ml glycitein, 0.2 mg/ml geistein (all from Sigma-Aldrich); 0.02 mg/ml 6'-O-malonly daidzin, 0.02 mg/ml 6'-O-malonly glycitin, 0.02 mg/ml 6'-O-acetyl daidzin, 0.02 mg/ml 6'-O-malonly genistin, 0.02 mg/ml 6'-O-acetyl glycitin and 0.02 mg/ml 6'-O-acetyl genistin (all from FUGICO Co., Ltd., Kitakyushu, Japan). The stock solution mixture was made into working standard solution mixtures of 6 concentrations to generate a standard curve (Table I)

Cell culture. The androgen-dependent human prostate cancer cell line LNCaP (Korean Cell Line Bank, Seoul, Korea) was obtained from Professor K.C. Choi (College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Thermo Fisher Scientific,

Table I. Concentration of working standard solution for HPLC analysis

	Standards (μg/ml)					
Isomer	1	2	3	4	5	6
Daidzin	4	8	16	32	64	128
Glycitin	2	4	8	16	32	64
Genistin	4	8	16	32	64	128
6'-*O*-malonyldaidzin	0.4	0.8	1.6	3.2	6.4	12.8
6'-*O*-malonylglycitin	0.4	0.8	1.6	3.2	6.4	12.8
6'-*O*-acetyldaidzin	0.4	0.8	1.6	3.2	6.4	12.8
6'-*O*-malonylgenistin	0.4	0.8	1.6	3.2	6.4	12.8
6'-*O*-acetylglycitin	0.4	0.8	1.6	3.2	6.4	12.8
Daidzein	4	8	16	32	64	128
Glycitein	2	4	8	16	32	64
6'-*O*-acetylgenistin	0.4	0.8	1.6	3.2	6.4	12.8
Genistein	4	8	16	32	64	128

Inc.), 10,000 U/ml penicillin G and 10,000 μg/ml streptomycin (Invitrogen, Thermo Fisher Scientific, Inc.) and 1% antifungal (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Invitrogen Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere of 5% CO_2 in air. Cells were detached with 0.25% trypsin-ethylenediaminetetraacetic acid (Invitrogen, Thermo Fisher Scientific, Inc.). Phenol red-free DMEM (Invitrogen, Thermo Fisher Scientific, Inc.) supplemented with 5 or 1% charcoal-dextran-treated FBS (CD-FBS) was used to block the effects of the estrogenic components of FBS and DMEM (12-15).

Cell proliferation assay. The inhibitory effects of SMD and its phytoestrogen components were assessed using a 3-(4-5-dimethylthiazol-2-yl)-2,5-dyphenyltetrazolium bromide (MTT) assay. LNCaP cells were plated at 4,000 cells per well in 96-well plates in 100 μl DMEM with 10% FBS. After incubation for 24 h, the medium was replaced with phenol red-free DMEM with 1% CD-FBS, followed by incubation for a further 48 h. Cells were washed with phosphate-buffered saline (PBS) and treated with vehicle [0.1% dimethyl sulfoxide (DMSO)], E2 ($2.7x10^{-7}$ mg/ml), GEN ($2.7x10^{-2}$ mg/ml), SMD (the lyophilized SMD powder was collected and then 3 g was dissolved in 1 ml of 100% methanol (Merck kGaA). The concentration of SMD was calculated by determining the concentration of total aglycon according to HPLC analysis, Table II, 0.79 mg/ml), casodex (CDX; also known as bicalutamide, an anti-androgen used to treat prostate cancer and hirsutism; $4.3x10^{-3}$ mg/ml), or ER antagonist ICI 182,780 ($6.1x10^{-6}$ mg/ml) in phenol red-free DMEM with 5% CD-FBS medium for five days. All of the above reagents were purchased from Sigma-Aldrich. To observe the effects on ER, CDX was applied in combination with each reagent to block the androgen receptor, while ICI 182,780 was used to block the ER. Media were changed every other day. At the end of the incubation period, 10 μl MTT solution (5 mg/ml; Sigma-Aldrich) was added to each well, followed by incubation for 4 h at 37˚C. The supernatants were then replaced with 100 μl DMSO (Sigma-Aldrich) to dissolve the formazan crystals. The optical density of each well was measured at 540 nm using an ELISA reader (SpectraMax® plus 384 absorbance microplate reader; cat. no. MNRO7122, Molecular Devices, Sunnyvale, CA,

Table II. Concentrations of aglycone isoflavones in SMD according to analytical high-performance liquid chromatography.

Isoflavone isomer	Concentration in SMD (mg/100 g)
Daidzin	27.057±0.002
6'-*O*-malonyldaidzin	18.616±0.026
6'-*O*-acetyldaidzin	21.410±0.281
Daidzein	1.988±0.411
Glycitin	25.471±0.030
6'-*O*-malonylglycitin	4.030±0.067
6'-*O*-acetylglycitin	3.764±0.037
Glycitein	23.537±0.010
Genistin	36.565±0.003
6'-*O*-malonylgenistin	1.216±0.080
6'-*O*-acetylgenistin	0.784±0.010
Genistein	0.685±0.054
Total isoflavone	165.123±0.629
Total malonyl	23.862±0.158
Total acetyl	25.958±0.291
Total aglycon	26.209±0.481
Metabolized daidzein[a]	39.803±0.431
Metabolized glycitein[a]	44.097±0.081
Metabolized genistein[a]	24.787±0.045

[a]Expected amount of aglycon metabolite generated by intestinal β-glucosidases in the body. This amount was calculated from the molecular weight and conversion coefficients. SMD, soy milk digestion extract.

Table III. Primer sequences and sizes for semi-quantitative reverse-transcription polymerase chain reaction.

Target gene	Sequences	Expected size
ERβ	Sense: 5'-TGTTACTGGTCCAGGTTCAAAGAGG-3' Antisense: 5'-AGCCACACTTCACCATTCCCAC-3'	200 bp
AR	Sense: 5'-CTC TCT CAA GAG TTT GGA TGG CT-3' Antisense: 5'-CAC TTG CAC AGA GAT GAT CTC TGC-3'	342 bp
PSA	Sense: 5'-TGCGCAAGTTCACCCTCA-3' Antisense: 5'-CCCTCTCCTTACTTCATCC-3'	754 bp
Cyclin D1	Sense: 5'-TCTAAGATGAAGGAGACCATC-3' Antisense: 5'-CCCTCTCCTTACTTCATCC-3'	354 bp
CDK4	Sense: 5'-TCGTGAGGTGGCTTTACTGA-3' Antisense: 5'-AGGCAGAGATTCGCTTGTGT-3'	698 bp
p21	Sense: 5'-AGGCACCGAGGCACTCAGAG-3' Antisense: 5'-TGACAGGTCCACATGGTCTTCC-3'	370 bp
GAPDH	Sense: 5'-ATGTTCGTCATGGGTGTGAACCA-3' Antisense: 5'-TGGCAGGTTTTTCTAGACGGCAG-3'	351 bp

USA) and was expressed as a percentage of the vehicle-treated group to represent the amount of viable cells.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was performed to determine the expression of selected target genes. LNCaP cells were cultured at a density of $3.5x10^6$ cells per well in a 60-mm round dish. Each reagent was applied at a concentration identical to that used in the cell proliferation assay. Total RNA was extracted at 0 and 24 h using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions and as described previously (12-15,30). The concentration of total RNA was measured using a SpectraDrop Micro-volume microplate (SpectraMax® plus384 absorbance microplate reader) at 260/280 nm. Complementary DNA (cDNA) was synthesized from total RNA using a power cDNA synthesis kit (iNtRON Biotechnology, Inc., Sungnam, Kyeonggido, Republic of Korea) for RT. cDNAs of ERβ, AR, PSA, cyclin D1, cyclin-dependent kinase (CDK)4, p21 and GAPDH were amplified using specific forward and reverse primer sets (Bioneer Corporation, Daejeon, Korea; Table III).

Taq polymerase, 10X PCR buffer, deoxynucleotide triphosphate mixture, $MgCl_2$ solution (Applied Biosystems, Thermo Fisher Scientific, Inc.) and cDNA template were used for PCR (Veriti 96 well Thermal cycler, Applied Biosystems) according to the manufacturer's instructions. PCR conditions were as follows: Denaturation step at 95˚C followed by 35 cycles of denaturation at 95˚C for 30 sec, annealing at 58˚C for 30 sec, and extension at 72˚C for 30 sec, with a final extension at 72˚C for 10 min. PCR products were run on a 2.0% agarose gel (Nusieve 3:1 Agarose; Lonza, Rockland, ME, USA) and gene bands were compared to 100-bp ladders (DAWINBIO, Hanam, Gyeonggi, Republic of Korea). Gels were scanned and the density of the bands was quantified using gel doc (Gel DocTM 2000 system, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All data were analyzed with GraphPad Prism 5.0 software (GraphPad Inc., La Jolla, CA, USA). Values are expressed as the mean ± standard deviation. Statistical analysis was performed using Student's t-test or one-way analysis of variance, followed by Dunnett's multiple comparisons tests. $P<0.05$ was considered to indicate a statistically significant difference between values.

Results

Concentration of isoflavone derivatives in SMD. To determine the concentration of total isoflavones, SMD was subjected to analytical HPLC. Twelve isoflavone isomers, including aglycones, β-glucosides, 7'-*O*-acetylglucosides and 7'-*O*-malonylglucosides, were confirmed in SMD (Table II). The concentration of total isoflavones in SMD was determined to be 165.123 mg/100 g, indicating a high abundance of this compound type. The concentrations of total 7'-*O*-acetylglucosides, 7'-*O*-malonylglucosides and aglycones were 23.862, 25.958 and 26.209 mg/100 g, respectively. Aglycones were most abundant among all isoflavones in SMD. The content of daidzein, glycitein and GEN was 1.988, 23.537, and 0.685 mg/100 g, respectively, with glycitein being the most abundant among the total aglycones. Next, an SMD solution with the same GEN content as that of the GEN solution used (0.027 mg/ml) was prepared in order to compare their effects. These results indicated that following digestion by bacterial β-glucosidases in the body, soy milk may have enhanced biological activities due to elevated aglycone contents. The expected aglycone concentrations following conversion with β-glucosidases were based on molecular weight and conversion coefficients (daidzin, 0.611; glycitin, 0.637; genistin, 0.625; M-daidzin, 0.506; M-glycitin, 0.534; M-genistin, 0.625; A-daidzin, 0.555; A-glycitin, 0.582; and A-genistin, 0.625).

SMD and its isoflavone components inhibit the proliferation of prostate cancer cells. To determine the anti-proliferative effects of SMD and its isoflavone component, an *in vitro* MTT assay was performed using the LNCaP prostate cancer cell line (Fig. 1A). The cell viability was significantly increased

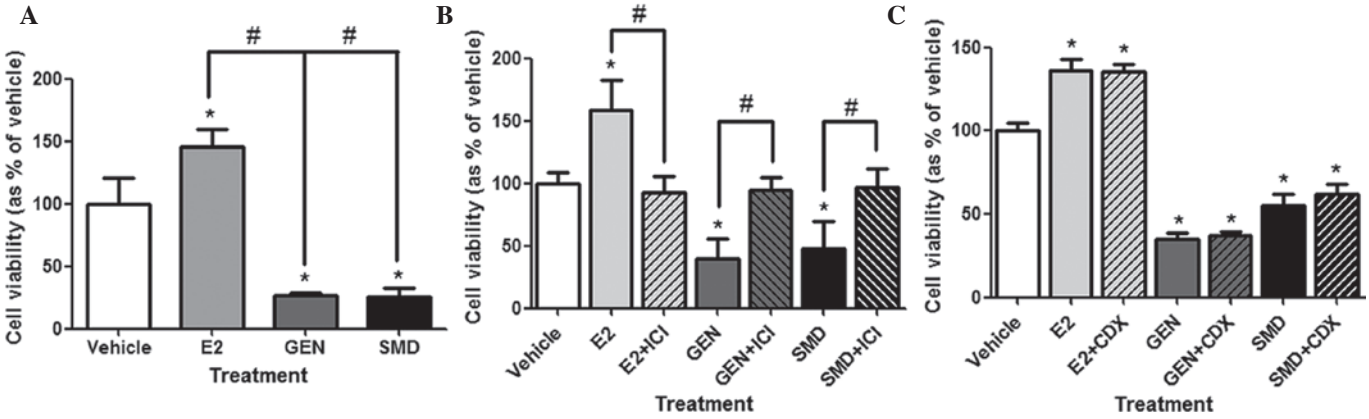

Figure 1. Effects of SMD and GEN in LNCaP cell proliferation via ER signaling pathway. (A) Inhibitory effects of phytoestrogens in SMD were analyzed using the MTT assay. Cells were treated with vehicle (0.1% dimethyl sulfoxide), E2 ($2.7x10^{-7}$ mg/ml), GEN ($2.7x10^{-2}$ mg/ml) or SMD (0.79 mg/ml) for five days. *P<0.05 vs. vehicle; #P<0.05 vs. E2 group. (B) To evaluate the effects of E2, GEN and SMD on cell growth in association with ER signaling, the ER antagonist ICI 182,780 was used. LNCaP cells were treated with vehicle, E2, GEN or SMD as above with optional co-treatment with ICI 182,780 ($6.1x10^{-6}$ mg/ml). (C) To determine whether or not the effects of E2, GEN and SMD are associated with the andogen receptor, cells were treated with CDX as above. LNCaP cells were treated with vehicle, E2, GEN or SMD as above with optional co-treatment with casodex ($4.3x10^{-3}$ mg/ml). Values are expressed as the mean ± standard deviation. *P<0.05 vs. vehicle; #P<0.05 as indicated. ER, estrogen receptor; SMD, soy milk digestion extract; E2, 17β-estradiol; GEN, genistein; CDX, carsodex (bicalutamide); ICI, ICI 182,780.

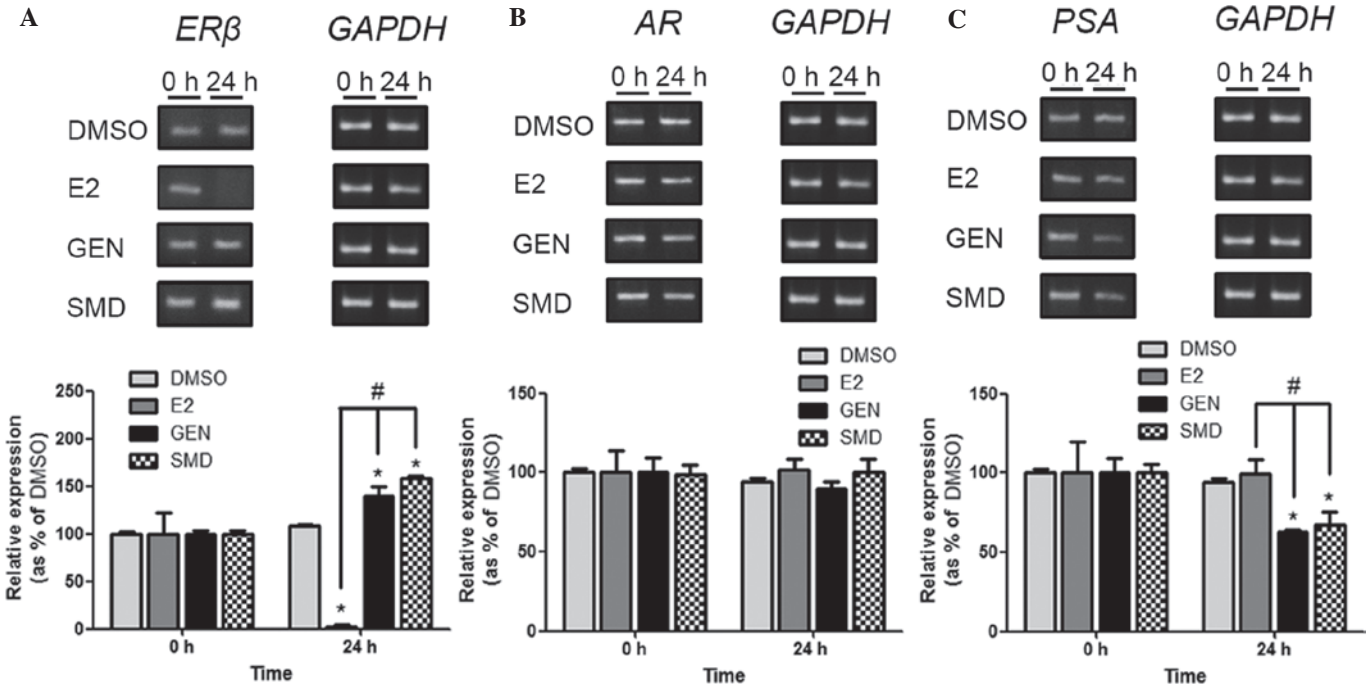

Figure 2. Effects of SMD on mRNA expression levels of (A) ERβ, (B) AR and (C) PSA genes. LNCaP cells ($3.5x10^6$/well) were treated with (0.1% DMSO), E2 ($2.7x10^{-7}$ mg/ml), GEN ($2.7x10^{-2}$ mg/ml) or SMD (0.79 mg/ml) and mRNA expression levels of target genes were determined by semi-quantitative reverse-transcription polymerase chain reaction analysis. Values are expressed as the mean ± standard deviation. *P<0.05 vs. DMSO group; #P<0.05 vs. E2 group. ER, estrogen receptor; AR, androgen receptor; PSA, prostate cancer-specific antigen; SMD, soy milk digestion extract; E2, 17β-estradiol; GEN, genistein; DMSO, dimethyl sulfoxide.

by 45±0.18% upon treatment with E2 compared with vehicle treatment (0.1% DMSO). However, GEN and SMD reduced the cell viability by 73.2±0.03 and 74.8±0.09%, respectively. These results showed that E2 has estrogenic activity in LNCaP cells expressing ERβ. Furthermore, GEN and SMD were demonstrated to inhibit LNCaP cell growth.

SMD inhibits prostate cancer cell growth through ER. To determine whether the effects of E2, GEN and SMD on LNCaP cell proliferation are mediated by ER signaling, the typical ER antagonist ICI 182,780 was applied in combination with E2, GEN or SMD (Fig. 1B). The increases in cell viability by E2 treatment and the decreases in cell viability by GEN and SMD treatment were completely abrogated by co-treatment with ICI 182,780. This result indicated that the cell proliferative effect of E2 as well as inhibitory effects of GEN and SMD are mediated via ER signaling.

To determine whether the effects of SMD and its isoflavone components are associated with the AR, cell proliferation was assessed with co-treatment with the anti-androgen CDX

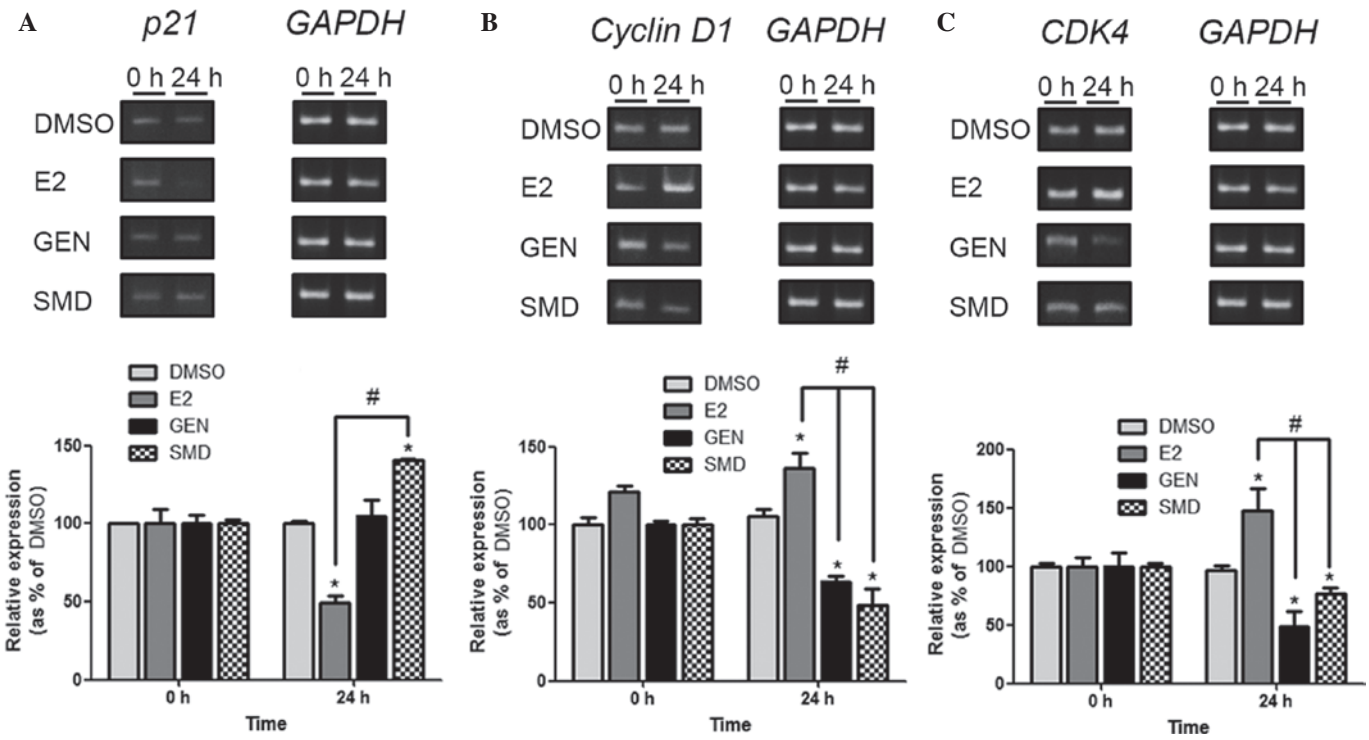

Figure 3. Effects of SMD on mRNA expression levels of cell cycle-regulatory genes (A) p21, (B) cyclin D1, and (C) CDK4. LNCaP cells ($3.5x10^6$/well) were treated with (0.1% DMSO), E2 ($2.7x10^{-7}$ mg/ml), GEN ($2.7x10^{-2}$ mg/ml) or SMD (0.79 mg/ml) and mRNA expression levels of target genes were determined by semi-quantitative reverse-transcription polymerase chain reaction analysis. Values are expressed as the mean ± standard deviation. *P<0.05 vs. DMSO group; #P<0.05 vs. E2 group. SMD, soy milk digestion extract; E2, 17β-estradiol; GEN, genistein; DMSO, dimethyl sulfoxide.

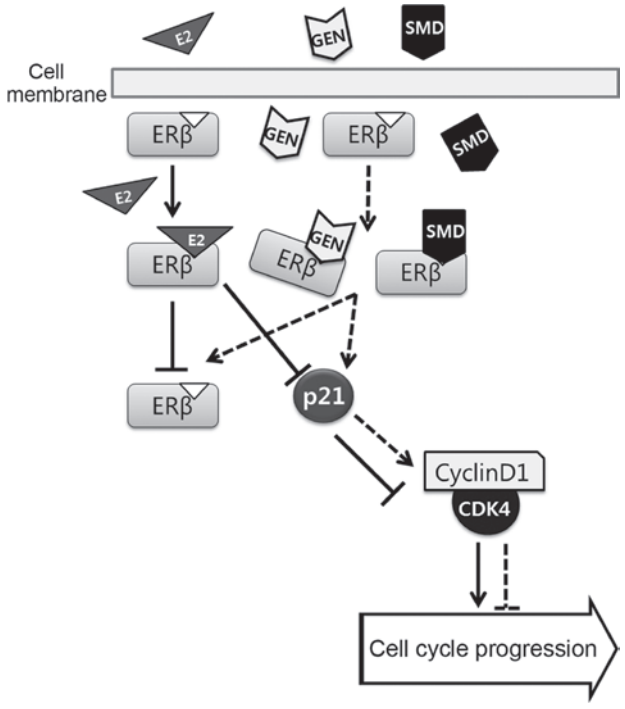

Figure 4. Role of SMD in the progression of E2-dependent LNCaP prostate cancer cells. LNCaP cells were treated with E2, GEN, and SMD. GEN, a phytoestrogen, and SMD, a combination of soy isoflavones, bound to ERβ due to structural similarity to E2. E2 reduced the translation of ERβ and p21, a tumor suppressor gene, thus increasing the cyclin-CDK complex. The cell cycle was promoted by E2 in LNCaP cells, while it was inhibited by GEN and SMD through ERβ, which bound to GEN and SMD, and then activated p21 to suppress the cyclin-CDK complex. As a result, SMD inhibited the growth of LNCaP cells by promoting the ERβ pathway and blocking cell cycle progression. SMD, soy milk digestion extract; E2, 17β-estradiol; GEN, genistein; CDK, cyclin-dependent kinase.

(Fig. 1C). E2-induced cell proliferation as well as the anti-proliferative effects of GEN and SMD were unaltered by CDX, suggesting that AR-associated signaling is not involved.

SMD activates ERβ and deactivates PSA in LNCaP cells. To determine the effects of SMD on the expression of ERβ, AR and PSA in LNCaP cells, RT-PCR analysis was performed (Fig. 2). The mRNA levels of ERβ were significantly reduced by E2, while SMD and GEN significantly increased ERβ expression compared with that in the DMSO-treated group. The expression of AR was unaltered by ER, GEN and SMD, indicating that they did not interact with the AR. The expression of PSA, which is an gene upregulated by androgens, was significantly reduced by GEN and SMD compared to that in the DMSO group, whereas it was unaltered by E2. These results suggested that E2-induced cell proliferation may be attributable to the inhibition of ERβ gene expression, and that the anti-cancer activities of GEN and SMD may be based on their ability to significantly enhance the expression of ERβ and reduce the expression of PSA.

SMD affects the mRNA expression of cell cycle-regulatory genes. The effects of SMD, GEN and ERβ on the mRNA expression levels of cell cycle-associated genes in LNCaP were then assessed by semi-quantitative RT-PCR on total RNA samples isolated from cells at 0 and 24 h. As shown in Fig. 3, the gene expression of Cyclin D1 and CDK4 was significantly elevated by E2, while being significantly reduced by GEN and SMD compared to that in the DMSO group. The mRNA expression levels of p21 were significantly elevated by SMD and reduced by E2, while remaining unaffected by GEN compared to the DMSO group. These results indicated that the gene

expression of Cyclin D1, a gene inducing cell cycle progression, was inhibited by the upregulated p21, a tumor suppressor gene, upon SMD treatment. The anti-proliferative effects of SMD on LNCaP prostate cancer cells are therefore likely to be based on affecting these signaling mechanisms of cell cycle regulatory genes.

Discussion

Soy milk, which is a product of whole soybeans, contains high amounts of protein, iron and niacin, and is free from cholesterol and lactose. In addition, soy-derived foods such as soy milk have been indicated to exert effects against osteoporosis as well as preventive effects against breast (31) and prostate cancers (23). Therefore, soy milk is an alternative to cow's milk with additional health benefits (18). Soy milk and soybeans are the most common and important dietary sources of isoflavones. Isoflavones, a sub-group of phytoestrogens, are diphenolic compounds present in plants, including soybeans, alfalfa, red clover and peanuts (32). The major isoflavones in soybeans are GEN, daidzein and glycitein. Soy isoflavones were demonstrated to have biological activities, including anti-oxidant and anti-inflammatory effects as well as anti-proliferative effects against cancer cells (33,34). While soy isoflavones and their metabolites have been indicated to be beneficial for the prevention or treatment of prostate cancer, the mechanisms of action have remained to be fully elucidated.

E2 (17β-estradiol) is a steroid hormone and exerts a strong estrogenic effect. In addition, it was reported to be linked to several types of cancer (12-15,25,26). GEN, a major isoflavone, is phytoestrogen and xenoestrogen, which influences multiple biochemical functions. E2 and GEN bind to the estrogen receptor, but result in opposing effects. E2 promotes the growth of cancer cells and GEN is reduces it (12,13). In a previous study, the growth of LNCaP human prostate cancer cells was significantly stimulated by E2 (35). In addition, E2 was shown to contribute to the risk of prostate cancer, whereas the phytochemical GEN derived from soy decreased the risk of prostate cancer (10). For this reason, the present study hypothesized that cell growth of prostate cancer cells was promoted by E2 and thus was used to compare the effects of isoflavones in the SMD. SMD contained a number of isoflavones, including GEN, according to the results of HPLC analysis. Therefore, it was hypothesized that GEN and SMD would have similar effects.

The present study examined the mechanisms underlying the preventative effects of SMD and its component GEN on prostate cancer cell proliferation and the expression of ERβ, which is associated with prostate cancer progression. The androgen-dependent LNCaP human prostate cancer cell line, which expresses ERβ at high levels, was adopted for evaluation of the tumor inhibitory effects of SMD along with E2 and GEN. The ERβ gene is expressed in prostate epithelial cells and has anti-proliferative and pro-apoptotic roles in cellular homeostasis (36). Soy isoflavones are structurally similar to E2 and are capable of binding to ERs (32). Soy isoflavones can be divided into three chemical groups: Aglycones [daidzein (4',7-dihydroxyisoflavone), GEN (4',5,7-trihydroxyisoflavone) and glycitein (4',7-dihydroxy-6-methoxyisoflavone)], β-glycosides [daidzin, GEN and glycitin], and further into 7'-*O*-acetylglucosides and 7'-*O*-malonylglucosides (37). Based on these facts, the present study hypothesized that soy isoflavones contained in SMD may bind to ERβ in LNCaP cells. Soy milk was digested and hydrolyzed using a pepsin suspension and pancreatin-bile salt mixture for extraction of aglycone isoflavones, which are bioavailable *in vitro*.

The presence of 12 isoflavone isomers was confirmed in SMD, including aglycone, and their concentration was determined. The effects of E2, GEN and SMD on LNCaP cell proliferation and ER signaling were evaluated using a cell proliferation assay and RT-PCR. The results showed that the viability and growth of LNCaP cells were promoted by E2, while being inhibited by SMD and GEN. Co-treatment with the ER inhibitor ICI 182,780 inhibited the effects of E2, GEN or SMD on the proliferation of LNCaP cells, whereas co-treatment with AR inhibitor CDX had no obvious effects. These results suggested that the effects of SMD are attributable to ER, but not AR signaling. In support of this, expression of ERβ was significantly elevated by SMD. mRNA expression of ERβ has been shown to be induced by SMD, and the combination of GEN, daidzein, and glycitein increases selectivity for ERβ binding. In addition, expression of PSA, which can be upregulated by androgens, was reduced by SMD, suggesting that SMD may potentially act as an anti-androgen.

The cell cycle has two major checkpoints: The G_1/S checkpoint, which controls entry into S phase, and the G_2/M checkpoint (38). Regulation of the cell cycle is closely linked to cancer cell proliferation and differentiation (14). Soy isoflavones of SMD can modulate the expression of genes that control the cell cycle and cell survival via the ERβ pathway, which may have inhibitory effects on G2 and M phases of the cell cycle (39). Cyclin D1 is a key regulator of cell cycle progression. Cyclins bind to their corresponding CDKs and form corresponding cyclin-CDK complexes. Cyclin D1 is known to function as a regulatory sub-unit of CDK4 or CDK6, whose activity is required for the G_0/G_1-S phase transition (12,14,38,40). In the present study, gene expression levels of Cyclin D1 and CDK4 were significantly elevated by E2 compared to those in the DMSO group, whereas they were considerably decreased by SMD. The tumor suppressor gene p21 directly regulates the G_1/S phase checkpoint by binding to cyclin-CDK complexes and leading to cell cycle arrest in G1 phase. In the present study, the expression of p21 was markedly increased by SMD. Therefore, SMD may potentially regulate the cell cycle through suppression of the Cyclin D1-CDK4 complex by upregulating p21 (Fig. 4).

In conclusion, the present study demonstrated that aglycone isoflavones from digested soy milk display inhibitory effects via ERβ signaling in LNCaP prostate cancer cells. SMD was found to contain diverse aglycone isoflavones produced by digestive processes and bacterial β-glucosidases, and inhibited the growth of LNCaP cancer cells by promoting the expression of ERβ and inhibiting the cell cycle. Therefore, soy milk may represent a nutriceutical for the prevention and inhibition of prostate cancer.

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