Involvement of the Estrogen Receptor β in Genistein-induced Expression of p21$^{waf1/cip1}$ in PC-3 Prostate Cancer Cells

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Abstract. *Background: Dietary genistein, a phytoestrogen derived from soybean, has been suggested as a chemopreventive agent for prostate cancer. Genistein has been reported to exert its anticancer effects via a variety of functional pathways, but the upstream signaling of molecules regulated by genistein remains unclear. In this study, estrogen receptor (ER) β involvement in genistein-induced expression of cell cycle inhibitors in PC-3 prostate cancer cells was investigated. Materials and Methods: The proliferation of PC-3 cells exposed to genistein was measured by the water-soluble tetrazolium salt (WST-1) proliferation assay. The expression of p21, p27 and ERβ in the PC-3 cells was assessed by quantitative real-time reverse transcription-PCR. ERβ silencing was performed using a small interfering RNA (siRNA). The transcriptional activity of the p21 promoter was determined by the luciferase reporter assay. Results: Genistein caused marked inhibition of proliferative activity and induced the expression of p21 and ERβ in the PC-3 cells. The siRNA against ERβ suppressed the genistein-induced expression of p21 and reduced the transactivation activity of the p21 promoter induced by genistein. Conclusion: ERβ is involved in genistein-induced expression of p21 in PC-3 cells.*

Epidemiologically, the incidence and mortality of prostate cancer appear to be significantly lower in Asian countries than in the USA and European countries (1, 2). Numerous studies have shown that the consumption of foods rich in soy isoflavones may be associated with a reduction in the risk of prostate cancer (3-5). Genistein, the predominant isoflavone in soybean, has strongly been suggested as an agent with some putative antitumor effects (6, 7). To date, several reports have suggested diverse mechanisms to explain the anticancer effects of genistein, including inhibition of protein tyrosine kinase (8), reduction of oxidative stress-inducing protein (9, 10), up-regulation of adhesion molecules (11, 12), inhibition of angiogenesis or cell migration (12), down-regulation of telomerase activity and DNA topoisomerase II (13-15) and induction of the expression of cell cycle inhibitors (16-19). Although genistein could exert its actions through a variety of molecular cascades as stated above, the details of the mechanism remain unclear.

Recently, the biological functions of two types of estrogen receptor, ERα and ERβ, in the prostate gland have been extensively studied. While ERα is predominantly expressed in the stroma of the normal gland, its expression level is very low in the epithelial cells; on the other hand, ERβ is mainly expressed in normal epithelial cells and prostate cancer cells (20). Interestingly, the expression level of ERβ is lower in precancerous lesions (*i.e.*, prostatic intraepithelial neoplasia), and elevated in metastatic lesions of prostate cancer (21-23). Several studies have suggested that ERβ may play a role in protection against malignant cell proliferation and distant metastasis (24, 25).

It has been reported that phytoestrogens have direct anti-tumor effects against prostate cancer, independent of the traditional pathway, namely, the pituitary-testicular axis. Potential candidates mediating these direct anticancer effects include ERβ (26). However, the downstream molecules regulated by ERβ, which might shed further light on the inhibitory effects of phytoestrogens against prostate cancer cells, have not yet been fully elucidated.

In this study, the effect of genistein on prostate cancer cell proliferation and the expression of cell cycle inhibitors and the possibility of ERβ association with the effects of genistein were examined using human PC-3 prostate cancer cells that show high levels of ERβ expression.

Materials and Methods

Cell culture and reagents. The human prostate cancer cells, PC-3, LNCaP and DU-145, were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The human normal prostate cells, PrEC and PrSC, were obtained from Cambrex Bio Science

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(Walkersville, MD, USA). The PC-3 and LNCaP cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The DU145 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The PrEC and PrSC cells were maintained in Prostate Epithelial Cell Growth Medium (PrEGM) SingleQuots and Stromal Cell Growth Medium (SCGM) SingleQuots (Cambrex), respectively. The genisitein was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO) for all the experiments. The final DMSO concentration was 0.1%.

Cell-proliferation assay. To evaluate the cell growth-inhibitory effect of genistein, the water-soluble tetrazolium salt (WST-1) assay (Premix WST-1 Cell Proliferation System, TaKaRa Bio, Shiga, Japan) was conducted. In brief, the PC-3 cells were seeded at $3x10^4$ cells/well onto 6-well tissue culture plates and incubated for 24 h. Then, the RPMI 1640 medium supplemented with 10% dialyzed (10,000 MW cut-off, Sigma) fetal bovine serum, penicillin and streptomycin and containing different concentrations of genistein was added, and the cells were incubated for an additional 144 h. Premix WST-1 was added to the medium and the cells were incubated for 3 h. Absorbance was measured at 450 nm. All the assays were carried out in triplicate.

Real-time PCR analysis of ERα and ERβ. The total RNA was isolated from the PrEC, PrSC, LNCaP, DU-145 and PC-3 cells using a RNAqueous Kit (Ambion Inc., Austin, TX, USA) in accordance with the manufacturer's instructions and reverse-transcribed into cDNA with random hexamers using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The cDNA was quantified by real-time PCR using the Prism 7300 Sequence Detection System (Applied Biosystems).

The PCR primers and TaqMan probes for ERα and ERβ (assay ID: Hs00174860_m1 and Hs00230957_m1, respectively) were purchased from Applied Biosystems. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (assay ID: No4352934E, Applied Biosystems) was used as the internal control for data normalization. To determine the absolute copy number of the target transcript, cloned plasmid DNA for ERα (pcDNA3- ERα) and ERβ (pcDNA3-ERβ) was used to generate a standard curve.

Real-time PCR for p21, p27 and ERβ. The total RNA was isolated from the PC-3 cells treated with different concentrations of genistein or vehicle for 24 h, and reverse-transcribed into cDNA. The cDNA was employed for TaqMan analysis in accordance with the instructions of the manufacturer.

PCR primers and TaqMan probes for p21 (assay ID: Hs00355782_m1) and for p27 (assay ID: Hs00153277_m1) were purchased from Applied Biosystems. GAPDH was used as the internal control for normalization. The obtained threshold cycle (CT) values were evaluated by the relative standard curve method and normalized using the respective values of the internal control.

ERβ silencing using small interfering RNAs (siRNAs) in PC3 cells and real-time PCR analysis for p21. The siRNA oligonucleotide against ERβ was synthesized by TaKaRa BIO. The sequences of the negative control siRNA (universal, TaKaRa BIO) used were 5'-UCUUAAUCG CGUAUAAGGCTT-3' and 5'-GCCUUAUACGC GAUUAAGATT-3'. The sequences of the siRNA used to knock-down ERβ expression were 5'-GUGUGAAGCAAGAUCGCUATT-3' and 5'-UAGCGAUCUUG CUUCACACTT-3'. The PC-3 cells were seeded at a density of $4.0x10^5$ in 100-mm culture plates in RPMI 1640 medium supplemented with 10% FBS. The following day, the cells were transfected with 50 nM of either the negative control siRNA (siControl) or the siRNA against ERβ (siERβ) using the X-tremeGENE siRNA Transfection Reagent (Roche, Mannheim, Germany), in accordance with the manufacturer's instructions. In the experiments conducted to assess the effect of genistein, the medium was removed 72 h after the transfection and the cells were washed once with PBS. Then, fresh medium supplemented with 10% dialyzed (10,000 MW cut-off, Sigma) fetal bovine serum was added to the cells, and the cells were treated with 100 μM genistein or DMSO (vehicle) for 24 h. Then, the total RNA was isolated and was used for real-time PCR analysis.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of ERβ transcript levels. The total RNA was extracted from the siRNA-transfected cells using a RNAqueous kit (Ambion), in accordance with the manufacturer's protocol. The total RNA (0.8 μg) from each sample was reverse-transcribed to cDNA using Avian Myeloblastosis Virus (AMV) Reverse transcriptaseXL (TaKaRa BIO). Semiquantitative RT-PCR was performed using TaKaRa Ex Taq HS (TaKaRa BIO). The primers used were 5'-TGAAAAGCAAGGTTAGTGGGAACC-3' and 5'-TGGTCAGGGA CATCATCATGG-3' for ERβ, and 5'-ACCACAGTCCATGCC ATCAC-3' and 5'-TCC ACCACCCTGTTGCTGTA-3' for GAPDH. The PCR consisted of 35 cycles for ERβ and 30 for GAPDH.

Transient co-transfection (siRNA oligonucleotide and reporter gene) and luciferase assay. The pKM2L-phP21 was purchased from RIKEN BioResource (Tsukuba, Japan); it contains a 2738-bp promoter region of human p21-cyclin dependent kinase inhibitor/WAF1/CIP1 ligated to the luciferase reporter gene. The pGL3 vector was purchased from Promega (Madison, WI, USA). The PC3 cells were seeded at $1x10^5$ cells/well onto 6-well tissue culture plates in RPMI 1640 medium supplemented with 10% FBS. After incubation for 48 h, the cells were transfected with 50 nM of either siControl or siERβ. After transfection with the siRNA for 72 h, the medium was removed and the cells were transiently transfected with 0.1 μg of the pKM2L-phP21 promoter reporter gene (renilla luciferase reporter) and 0.1 μg of the pGL3 vector (firefly luciferase reporter as the internal control) using the FuGENE transfection reagent (Roche), in accordance with the manufacturer's instructions. On the following day, genistein was added at various concentrations to the incubation medium and the cells were incubated for an additional 24 h. Cell lysates were obtained using the cell lysis buffer provided in the Dual Luciferase assay system (Promega), and the luciferase activities were measured using a luminometer. The p21 promoter activity was normalized using the corresponding firefly luciferase reporter activity.

Statistical analysis. Data analysis was performed using Student's *t*-test. $P<0.01$ was considered to denote statistical significance.

Results

Inhibition of cell proliferation by genistein. As shown in Figure 1, genistein caused marked inhibition of the proliferation of the PC-3 cells in a dose-dependent manner. The cell-growth was reduced to 50, 45 and 20% of that in the control by genistein at concentrations of 50, 100 and 200 μM, respectively.

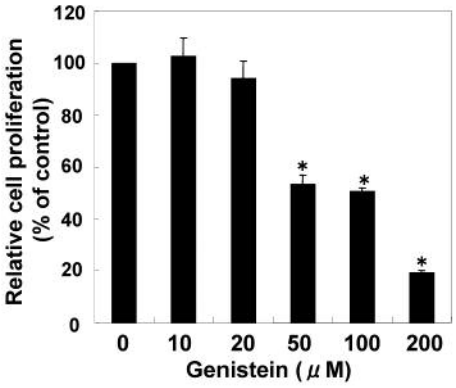

Figure 1. *Effects of genistein on the growth of the PC-3 cells. PC-3 cells were treated for 144 h with various concentrations of genistein as indicated. Cells treated with vehicle (0.1% DMSO) were used as the control . The number of viable cells treated with genistein or DMSO for 144 h was determined by the WST-1 assay. Three individual experiments were performed and means±SD are presented; *p<0.01 as compared to control (Student's t-test).*

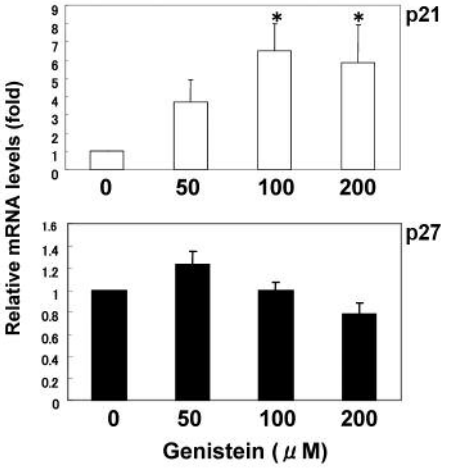

Figure 2. *Gene expression levels of p21 and p27 after incubation with genistein in PC-3 cells. PC-3 cells were incubated with genistein (50, 100 or 200 μM) or vehicle (control, 0.1% DMSO) for 24 h. The gene expression levels of both were estimated using the Taqman real-time PCR. Three individual experiments were performed. Values are means±SD of the gene expression level relative to that in the control, which is given an arbitary value of 1. *p<0.01 (Student's t-test).*

Induction of expression of the cell cycle inhibitor p21 by genistein. As shown in Figure 2, the expression level of p21 mRNA assessed by real-time PCR was clearly increased in genistein-treated PC3 cells as compared with that in the control. On the other hand, the p27 expression level was not altered significantly.

Expression of ERα and ERβ in human normal prostate cells and prostate cancer cells. As shown in Figure 3A, the expression levels of ERα in the prostate cancer cell lines examined (LNCaP, DU-145 and PC-3) were very low as compared with those in the normal prostate stromal cells, PrSC, and normal prostate epithelial cells, PrEC. With respect to the expression pattern of ERβ, the PC-3 cells expressed ERβ at the highest level among the cell lines (Figure 3B).

Enhancement of ERβ expression by genistein. Exposure to genistein for 24 h up-regulated the expression level of ERβ mRNA in the PC-3 cells, and the effect was maximal following treatment with 100 μM genistein (Figure 4). The increase of ERβ expression by different doses of genistein was similar to the effect of the compound on the expression of p21.

Effect of the siRNA against ERβ (siERβ) on the genistein-induced expression of p21. The influence of ERβ on the induction of p21 expression by exposure of the PC-3 cells to genistein was examined using the RNA interference technique. The efficacy of siERβ was determined by evaluating the degree of knock-down of the ERβ transcript as compared with that in the control (siControl) using RT-PCR. As shown in Figure 5A, the amount of the ERβ transcript was reduced remarkably in the PC-3 cells transfected with an siERβ as compared with that in the control PC-3 cells. Knock-down of the ERβ transcript significantly inhibited the enhancement of p21 expression in the PC-3 cells treated with 100μM genistein (Figure 5B).

Effects of the siRNA against ERβ (siERβ) on the p21 promoter activity. The influence of ERβ on the transcriptional activity of the p21 promoter in the PC-3 cells treated with genistein was investigated by co-transfection with the wild-type p21 promoter plasmid and an siERβ. The effects were assessed by measurement of the luciferase activity. Knock-down of ERβ clearly inhibited transcriptional activity of the p21 promoter in the PC-3 cells exposed to various concentrations of genistein (Figure 6).

Discussion

Our current experiments revealed that genistein induced marked suppression of prostate cancer cell proliferation and that it enhanced the expression of p21, a cell cycle inhibitor, which are consistent with previous reports (17, 18).

Recently, attention has been focused on ERβ as a candidate mediating the antitumor actions of antiestrogens/ selective estrogen receptor modulators (SERMs) and phytoestrogens against prostate cancer cells (21, 25). In the present study, the induction of p21 expression by genistein in PC-3 cells that show high levels of expression of ERβ was significantly

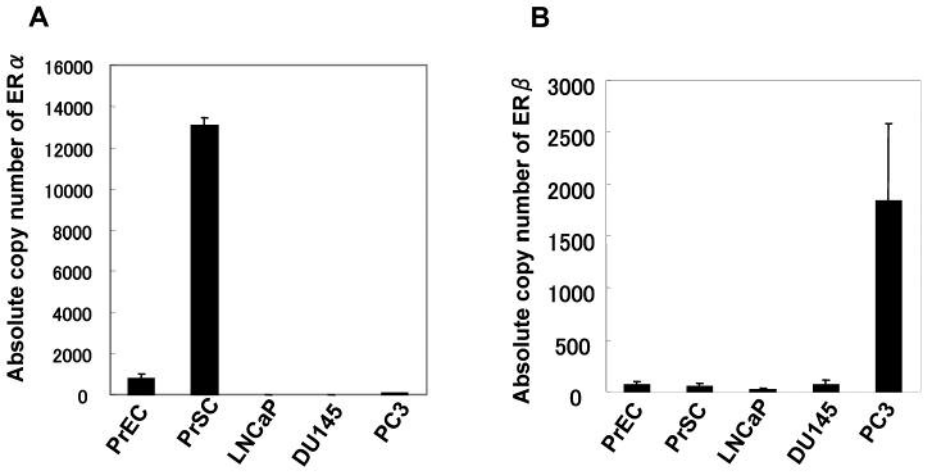

Figure 3. *Expression of ERα (A) and ERβ (B) mRNA in various human prostate cell lines. Transcript levels of both ERs are shown as the copy number normalized by the expression levels of the human GAPDH gene. Three independent experiments were performed.*

suppressed by knock-down of the ERβ transcript using an siRNA. Furthermore, the transcriptional activity of the p21 promoter activated by genistein was inhibited by transfection of the siRNA against ERβ. These results support the notion that ERβ is a major regulator of genistein-induced p21 transcription in PC-3 cells. The regulation of p21 expression in the PC-3 cells was independent of p53-mediated signaling, because PC-3 cells are deficient in p53 expression (27). To the best of our knowledge, this report is the first to exhibit an association between genistein-induced p21 expression and ERβ.

Most phytoestrogens, including genistein, have weak affinities for both ERα and ERβ, although they tend to bind more strongly to ERβ (28). Additionally, they have been shown to recruit transcriptional co-factors more actively with ERβ than with ERα (29). The two ERs exert transactivation activity *via* several enhancer elements, including the estrogen response element (ERE), the activating protein-1 (AP-1) site, the cyclic AMP-response element, the Sp1 element and the nuclear factor (NF)-kappa B site in the promoter regions of the target genes after binding to different ligands (30-33). In the proximal promoter region of p21, sequences corresponding to the AP-1, Sp1 and NF-kappa B binding sites, but not ERE, have been detected using the TRANSFAC database (http://www.cbrc.jp/research/db/TFSEARCH.html). Hence, ERβ bound to genistein induces p21 expression possibly through binding to these cis-elements. Further experiments are necessary to identify the ERβ-binding site on the promoter region of p21.

The effect of the knock-down of ERβ on proliferation of the PC-3 cells was also examined. The genistein-induced suppression of cell growth was not significantly reversed by suppressing the expression of ERβ using the siRNA (data not shown). The anticancer effect of genistein mediated by

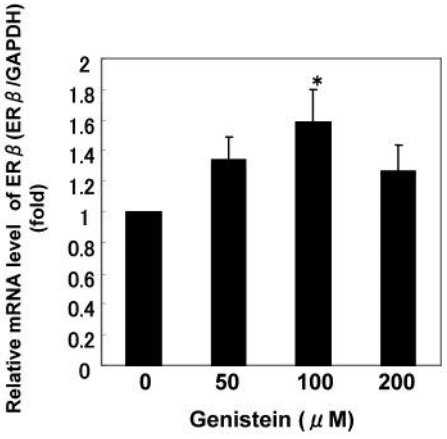

Figure 4. *Gene expression of ERβ after incubation with genistein in PC-3 cells. The levels of the ERβ transcript were determined by Taqman real-time PCR. PC-3 cells were incubated with genistein (50, 100 or 200μM) or vehicle (control; 0.1% DMSO) for 24 h. Three individual experiments were performed. Values are means±SD of the gene expression level relative to that in the control, which is given an arbitary value of 1. *p<0.01 (Student's t-test).*

the ERβ might contribute, in part, to the inhibition of cell proliferation in the PC-3 cells, because genistein is known to act *via* diverse functional pathways. It has been reported that apigenin, one of the flavonoids, is capable of suppressing cell growth of both DU-145 prostate cancer cells and MDA-MB-231 breast cancer cells by activating caspase-3 *via* the ERβ pathway (26). In that report, the knock-down of ERβ expression also did not reverse genistein- induced inhibition of cell growth (26). Further studies are necessary to elucidate the molecular mechanisms underlying the suppression of the growth of cancer cells by phytoestrogens.

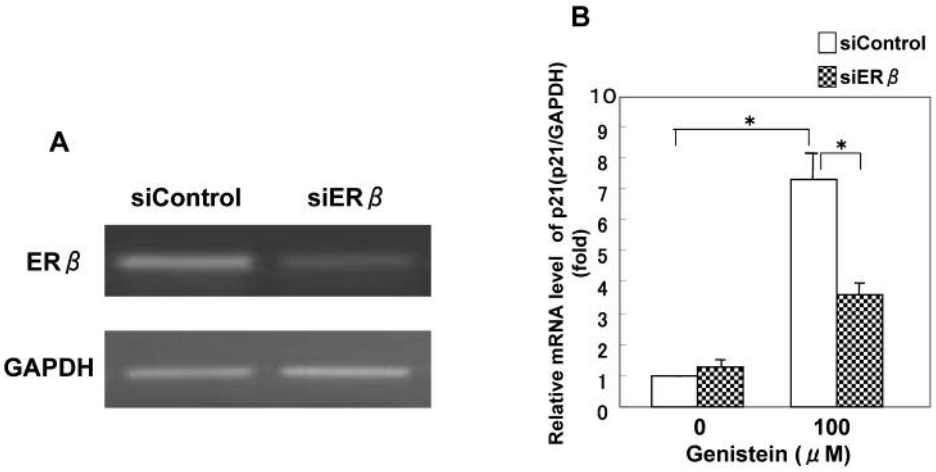

Figure 5. *(A) Effect of siRNA transfection on ERβ expression. PC-3 cells were transfected with 50 nM siERβ or siControl, as described in the materials and methods section. At 72 h after the transfection, total RNA was extracted and semiquantitative RT-PCR was performed using primers against ERβ and GAPDH. (B) Effect of siRNA transfection on p21 expression. PC-3 cells were transfected with siERβ or siControl for 72 h, followed by administration of either genistein (100 μM) or DMSO (vehicle) for 24 h. The expression levels of p21 were analyzed by real-time PCR analysis and corrected for the GAPDH level as the internal control. Values measured with the siControl and DMSO were defined as 1 (control). Results (means±SD) are representative of three independent experiments. *p<0.01, as compared to the control (Student's t-test).*

In prostate cancer, the expression level of ERβ has been shown to decrease with progression of the tumor grade, but to be increased at metastatic sites (22, 23). It has been reported that the regulation of methylation in the ERβ promoter sequences may be linked to the pattern of ERβ expression (34).

Regulation of ERβ-mediated transactivation by some agents, including phytoestrogens and antiestrogens/ SERMs, might yield anticancer effects against advanced prostate cancer, for which no curative therapy has yet been established.

In conclusion, ERβ is involved in genistein-induced p21 expression in PC-3 cells.

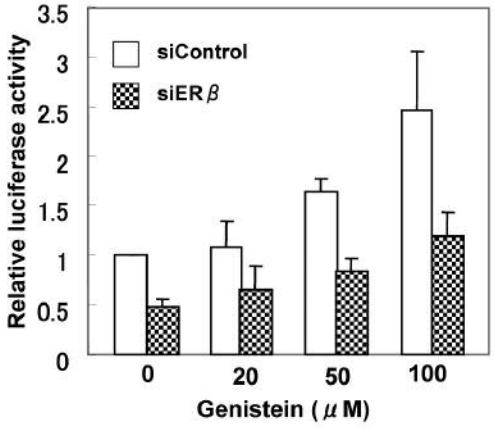

Figure 6. *Inhibition of p21 promoter activities by RNA interference technique in the PC-3 cells. PC-3 cells were transfected with 50 nM of either control siRNA or ERβ siRNA for 72 h and then transfected with 0.1 μg of the pKM2L-phP21 promoter reporter gene and 0.1 μg of the pGL3 vector (as the internal control) for 24 h. On the following day, genistein was added at various concentrations. The relative luciferase activities were measured using the Dual-luciferase reporter assay system after incubation for 24 h. The values represent the means±SD from three independent experiments.*

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