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Yak-kong and Soybean Induced Expression of Osteoprotegerin in MG-63 Human Osteoblastic Cells Requires Estrogen Receptor-β

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Phytoestrogens, especially Yak-kong or soybean-derived isoflavones have been traditionally used as a supplement of estrogen for preventing postmenopausal osteoporosis in oriental folk medicine. In our previous study, the treatment of Yak-kong and soybean increased estrogen receptor-α (ERα) expression and proliferation of MG-63 osteoblastic cells. In contrast, the increase of estrogen receptor-β (ERβ) expression in proliferating MG-63 cells with Yak-kong and soybean treatment was less pronounced, which suggested that ERβ may play a role rather in the regulation of bone cell differentiation. To determine the role of ERβ in Yak-kong or soybean mediated regulation of bone cell differentiation, we established MG-63 cell lines stably expressing either ERβ or antisense ERβ RNAs. Increased expression of ERβ did not affect ERα expression and proliferation of MG-63 cells. However, increased expression of ERβ in MG-63 cells (ERβ-MG63 cells) selectively enhanced Yak-kong or soybean induced expression of osteoprotegerin (OPG), a novel soluble glycoprotein which is secreted from osteoblasts and mediates the signal for osteoclast differentiation. Inhibition of ERβ expression by antisense ERβ RNAs (As-ERβ-MG63) caused these cells to insensitize Yak-kong or soybean induced expression of OPG but increased MG-63 cell proliferation. Furthermore, the comparable effects between Yak-kong and the combined treatment of genistein and daidzein at 0.5×10^{-8} M, which is a concentration of these two isoflavones similar to Yak-kong at 0.001 mg/mL, on OPG expression in ERβ-MG63 cell demonstrate that the enhanced expression of OPG with Yak-kong treatment is mediated by the synergistic effect of low leveled isoflavones in the extracts. Together, coupled with low level of ER expression in osteoclasts, our data demonstrate that ERβ in osteoblasts plays an important role in Yak-kong and soybean mediated inhibition of osteoclast differentiation indirectly by enhancing the expression of OPG.

Key words : Estrogen receptor β, Osteoprotegerin, Yak-kong, Soybean, MG-63 cells

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INTRODUCTION

Osteoporosis is a bone disease characterized by decreased bone density without chemical composition changes and increased frequency of fracture. At the cellular level, it is due to decreased bone formation by osteoblasts and increased bone resorption by osteoclasts.[1,2] Both men and women experience bone loss with osteoporosis due to the increased action of osteoclasts during the process of aging, but menopausal women have particularly high incidents of osteoporosis because of decreased secretion of female hormone, estrogen, after menopause.[3] Estrogen replacement therapy has been used for the treatment of osteoporosis, but its inhibitory effect on bone loss was lower in the age group over 60 than in the group at early menopause. It also requires at least 5 years of long-term treatment,[2] and has adverse effects such as irregular uterine bleeding, breast cancer, corpus carcinoma, and increased hypertension.[3] Thus researches on alternative therapies using natural active ingredients in Oriental herb medicine or foods have been actively investigated to complement these risks.[4-8]

Alternative therapy for the prevention and treatment of osteoporosis in postmenopausal women includes the oral administration of phytoestrogens or intake of foods containing large amounts of these substances.[5,9] Phytoestrogens are divided into three groups of isoflavones, coumestans, and lignans, among which isoflavones are representing phytoestrogens that have been actively studied in relation to bone metabolism,[5,9] and 12 types have been known including aglycones such as genistein, daidzein, and glycitin, to which sugars are bound to form glycosides.[5,9] Among these substances, daidzein and

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genistein have similar structures with estrogen and thus are bound to estrogen receptor (ER) such as ERα or ERβ inside the cell[10] and moved to the nucleus, and then recognizes estrogen response element (ERE) of the gene that is involved in bone metabolism and regulates its expression. While estrogen has high affinity to ERα receptor, daidzein and genistein have more than 5 times higher affinity to ERβ than to ERα and thus these substances have been highlighted as alternative substances for the prevention of osteoporosis because they show not only estrogenic effect but also anti-estrogenic effect through the binding to ERβ without inducing several adverse effects of estrogen replacement therapy.[5,9]

Phytoestrogens are largely contained in alfafa, pea, oatmeal, red bean, rice, and soybean, and particularly isoflavones are abundantly found in beans and legumes.[11] While soybeans, which contain large amount of isoflavones, have been widely used in western countries and Korea,[5] Yak-kong (*Rhynchosia volubilis*, 鼠目太) has been generally used in Oriental medicine or folk remedy.[11,12] In previous studies for analyzing isoflavone contents, the contents of daidzein and genistein from Yak-kong extract were higher than that of soybean,[11,13,14] and when treated on MG-63 human osteoblastic cells, both Yak-kong extract and soybean extract increased the cell proliferation and selective expression of IGF-I.[15,16] Also, Yak-kong extract and soybean extract treatments significantly induced the expression of ERα, an estrogen receptor, in addition to the increased proliferation of MG-63 osteoblastic cells,[15,16] and more selective expression of IGF-1 and cell proliferation were significantly increased in MG-63 osteoblastic cells with constant ERα expression by Yak-kong or soybean extract treatment.[17] However, the increase of ERβ expression by isoflavone treatment in the proliferating MG-63 osteoblastic cells was not significant.[15,16,18] These results suggest that ERβ has other roles such as osteocytes differentiation in bone metabolism in addition to induced cell proliferation in osteoblastic cells. Therefore, this study was performed to produce MG-63 human osteoblastic cell line with increased ERβ expression (ERβ-MG63 cell line) and MG-63 human osteoblastic cell line with extremely suppressed ERβ expression (As-ERβ-MG63 cell line) with the method of molecular biology, and to measure the changes in the expression of differentiation factors that are secreted from osteoblasts and influence the osteoclast differentiation after the treatment of Yak-kong or soybean extract to each cell line, and to examine the role of ERβ for the expression of differentiation factors by Yak-kong or soybean treatment in MG-63 osteoblastic cells.

MATERIALS & METHODS

1. Materials and Reagents

MG-63 human osteoblastic cells were obtained from the Korean Cell Line Bank (College of Medicine, Seoul National University) and used in the laboratory by subculturing. For standard materials, 17-estradiol (E_2), genistein, and daidzein (Sigma Chem. Co.) were used. Methanol for extraction was obtained from Merck (Damstandt, Germany) and other experimental reagents used were reagent-grade. FBS was purchased from United Biotechnology (Saranac Lake, NY). ERα, ERβ and IGF-I antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA).

2. Extraction and Sample Preparation of Yak-kong and Soybean

Yak-kong used in this study was produced and harvested at Jeongseon in 2002 (purchased at Gyeongdong Market) and soybean used in this study was produced and harvested at Yangju, Gyeonggi-do in 2002 (Hansalm Foods Co.). Each 1 kg of Yak-kong or soybean was extracted with 5 times volume of 70% methanol and filtered, and the remaining liquid was evaporated and concentrated and then freeze dried for 72 hours to produce Yak-kong methanol extract (containing 1.12% isoflavone) and soybean methanol extract (containing 0.69% isoflavone). Yak-kong or soybean methanol extract was dissolved in dimethylsulfoxide (DMSO, Sigma Chem, Co., final concentration <0.05%, v/v) and filter-sterilized with a filter (0.22 m pore size, Millipore) before use.

3. Human Osteoblastic Cell Culture

MG-63 human osteoblastic cells were adhered to polystyrene cell culture plates and incubated by using 1% antibacterial-antifungal solution containing penicillin and streptomycin (Gibco, USA) and DMEM (Gibco, USA) with 10% FBS (United Biotechnology, USA) added.[19] During the incubation, the humidity and temperature were maintained at 95% and 37℃, with constant supply of 5% CO_2.

For the exclusion of influences by estrogenic substances contained in FBS and DMEM culture solutions,[20] Yak-kong extract or soybean extract treatment was performed on MG-63 human osteoblastic cells that were incubated with 60% confluency in phenol red-free MEM containing 10% charcoal-striped FBS. For the positive control, each single standard substance of E_2, daidzein and genistein and combined standard substances of daidzein and genistein were treated under the same

culture condition. As described in the previous studies[15,16] for positive controls, each 10^{-9} M and 10^{-6} M of E_2, daidzein and genistein single standard substance and 0.5×10^{-8} M and 0.1×10^{-8} M of daidzein and genistein combined standard substances were dissolved in dimethylsulfoxide (DMSO, Sigma Chem. Co., final concentration <0.05%, v/v). The concentrations of Yak-kong and soybean extracts were decided as 0.001, 0.01, and 0.1 mg/mL as used in the previous studies.[15,16]

The concentration of each standard substance was decided on the basis of blood isoflavone level of 50~800 ng/mL ($0.2\sim3\times10^{-6}$ M) of an adult whose food intake contained 50 mg of isoflavone a day and blood estrogen level of 40~80 pg/mL ($0.15\sim0.3\times10^{-9}$ M) of an adult female.[9] The concentrations of combined standard substances of daidzein and genistein were decided as 0.5×10^{-8} M/each and 0.1×10^{-8} M/each as examined in the previous studies on the basis of concentrations of daidzein and genistein in 0.001 mg/mL Yak-kong extract or soybean extract (Yak-kong extract contained daidzein 0.35×10^{-8} M and genistein 0.4×10^{-8} M; soybean extract contained daidzein 0.2×10^{-8} M and genistein 0.04×10^{-8} M).[11,13,14]

4. ERβ Plasmid & Antisense ERβ Plasmid

The *Not*I-*Hind*III fragment of 1.8 kb human ERβ cDNA[21] was subcloned into pcDNA3 vector and then the sense and antisense orientations were identified by using *Bgl*II as a restriction enzyme to produce sense and antisense pcDNA3-ERβ plasmids (pcDNA3-ERβ: pcDNA3-As-ERβ).

5. Stable Transfection of Sense and Antisense ERβ

MG-63 osteoblastic cells were plated at the concentration of 2×10^5 cells/35 mm plate, and pcDNA3-ERβ plasmid (1 μg) or pcDNA3-As-ERβ plasmid (1 μg) was mixed with 2.5 M $CaCl_2$, 2×HEPES and reacted by using DNA-Calcium phosphate coprecipitation method.[22] For the control cells, only pcDNA3 vector (1 μg) was mixed with 2.5 M $CaCl_2$, 2×HEPES and then proceeded with the same method.[22] After 16 hours, cells were washed with PBS containing 15% glycerol and treated with the culture solution containing G418 (250 μg/mL) antibiotics for 4 weeks, and then only pcDNA3 (vector only: control), pcDNA3-ERβ or pcDNA3-As-ERβ transfected cells were selected and the ERβ expression on each cell strain was confirmed by western blot hybridization.

6. Measurement of Human Osteoblastic Cell Proliferation

Measurement of osteoblastic cell proliferation was performed by using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] colorimetric assay (Cell Titer 96 Aqueous One solution Cell Proliferation Assay: Promega) that allowed massive searches in short period of time.[15] Control MG-63 cells, ERβ-MG63 cells or As-ERβ-MG63 cells were plated on 96 well plates at the concentration of 0.5×10^4 cells/well and left for 24 hours, and then Yak-kong methanol extract, soybean methanol extract, single standard substance of E_2, daidzein, and genistein, and combined standard substances of daidzein and genistein were applied by concentration and incubated for 5 days. At day 5, MTS (20 ul/well) reagent was added and incubated for 4 hours at 37 ℃, and the amount of MTS that degraded to formazan was decided by measuring the absorbance at 490 nm using Elisa Reader. Each treatment group of 10 wells was prepared and the experiment was repeated 3 times, and the effect of cell proliferation for each agent was obtained as the average value from 3 repeated experiments and presented as percentage for the DMSO only treated control group.

7. Western Blot Hybridization

The expressions of estrogen receptor α and β (ERα: ERβ) and proliferation (Insulin Like Growth Factor I: IGF-I) and differentiation (Osteoprotegrin: OPG; Tumor Necrosis Factorα: TNFα) related local factors were examined by using western blot hybridization.[15,16] Control MG-63 cells, ERβ-MG63 cells or As-ERβ-MG63 cells treated with Yak-kong methanol extract, soybean methanol extract, and each standard substance for 5 days were mixed and dispersed in 1ml of RIPA buffer (20 mM Tris-HCl, pH 8, 150 mM $NaCl_2$, 10 mM $NaPO_4$, 10% glycerol, 100 M Na_3VO_4, 100 M ammonium molybdate, 1% NP-40, 0.1% SDS) at 4 ℃, and cell walls were disrupted for 5 seconds using an ultrasonicator at low intensity and stored at -20 ℃ for 16 hours. After thawing, the homogenate was centrifuged at 1500×g for 5 minutes and the supernatant was prepared as protein extract. Protein samples for the expression of local factors that were synthesized and secreted from osteoblasts and affected osteoclasts, were prepared by concentrating MG-63 cell culture solution, which was treated with Yak-kong methanol extract, soybean methanol extract, and each standard substance for 5 days, with Centriplus Column (Amicon Cat No. 4420). The protein content was decided by measuring the absorbance at 595 nm in the Bio-Rad protein assay with bovine serum albumin as a standard.

Prepared protein was mixed with 2× sample buffer and boiled for 5 minutes at 95 ℃, and placed on 10% poly-

acrylamide/SDS gel for electrophoresis and then adsorbed to Hybond ECL nitrocellulose membrane. Primary antibodies (ERα, ERβ, IGF-I, OPG, TNFα: Santa Cruz Biotech, Inc, USA) were diluted in PBS containing 5% skim milk and 0.1% Tween 20 and reacted at 4 ℃ for 16 hours, and then washed with PBS containing 0.01% Tween-20 for 15 minutes for 3 times. Peroxidase-conjugated anti-IgG as secondary antibody was diluted 1:5000 in blocking solution and reacted at room temperature for one hour and washed with PBS containing 0.1% Tween-20 for 3 times. The color formation was confirmed by using ECL hyperfilm and the signal intensity was quantified using the imaging densitometer (model GS-700, BIO-RAD, USA) and presented as percentage for the DMSO only treated control group. Western blot hybridization for each of ERα, ERβ, IGF-I, OPG, and TNFα was repeated 6 times to present the most representing results.

8. Statistical Analysis

Experimental results were analyzed by using the SAS statistical program (SAS institute, 1987) and presented as mean±SE. The significance between control group and treatment group on the proliferation of osteoblastic cells and the changes in the expression of ERα and ERβ, and IGF-I was verified using Duncan's multiple range test of the general linear model (GLM) at the level of $p<0.05$.

RESULTS & DISCUSSION

1. Changes in the ERβ and ERα Expression by Stable Transfection

Each of pcDNA3-ERβ and pcDNA3-As-ERβ plasmid was transfected into MG-63 cells and treated with G418 antibiotics for 4 weeks, and then cells with resistance were pooled and the changes of ERβ expression were examined by western blot (Fig. 1). When compared to control MG-63 cells transfected by pcDNA3 vector only (100%) (Fig. 1A and C: lane 1), pcDNA-ERβ transfection increased the ERβ expression to 360.2% but pcDNA3-As-ERβ transfection decreased the ERβ expression to 63.8% (Fig. 1A and C: lane 2, 3). While in the control, ERβ-MG63 and As-ERβ-MG63 cells, the degree of ERα expression was not significantly different (Fig. 1B and C). This means that pcDNA-ERβ and pcDNA3-As-ERβ plasmid transfection on MG-63 cells did not influence the ERα expression.

2. Changes in the Expression of Estrogen Receptors in ERβ-MG63/As-ERβ-MG63 Osteoblastic Cells by Yak-kong or Soybean Treatment

The effect of Yak-kong or soybean extract treatment in addition to each standard substance of estrogen, daidzein, and genistein in ERβ-MG63 and As-ERβ-MG63 cells on the expression of ERβ and ERα was examined

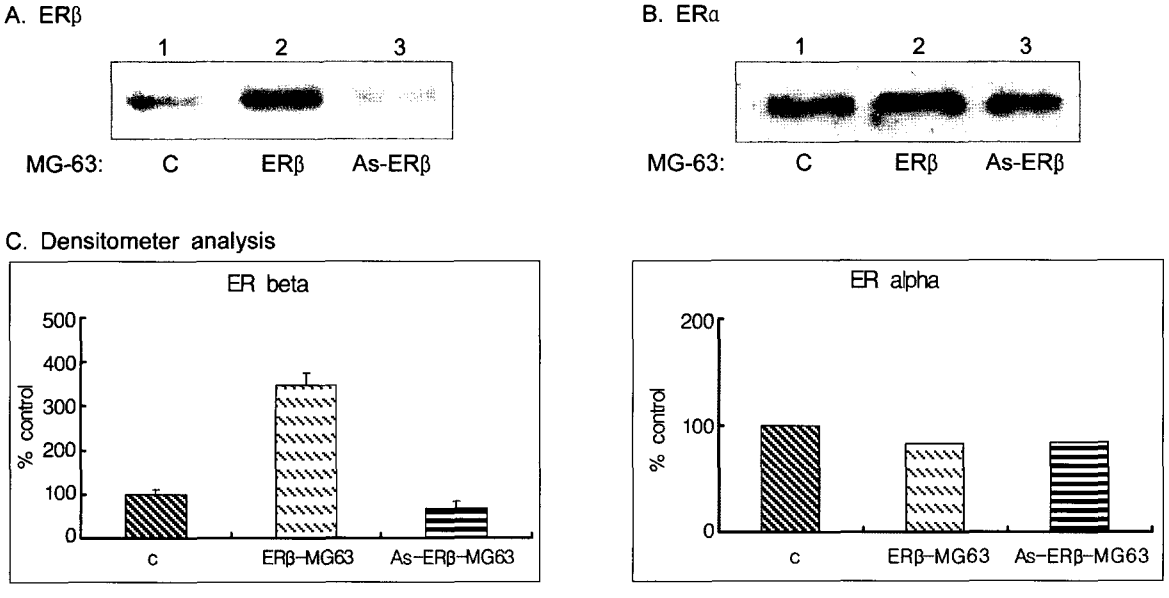

Fig. 1 ERβ and ERα expression in transfected MG-63 cells.

A and B. Protein extracts (10 μg/lane) from control (vector pcDNA3 only: lane 1), pcDNA3-ERβ (lane 2) or pcDNA3-As-ERβ (lane 3) transfected MG-63 cells were subjected to SDS-PAGE and immunoblotting with ERβ (A) or ER α (B) specific antibodies.

C. Films from A and B were scanned with a densitometer and the area of the resulting peaks were normalized, first to the corresponding value of actin (not shown) and then calculated to the area value of control MG-63 cell (100%)

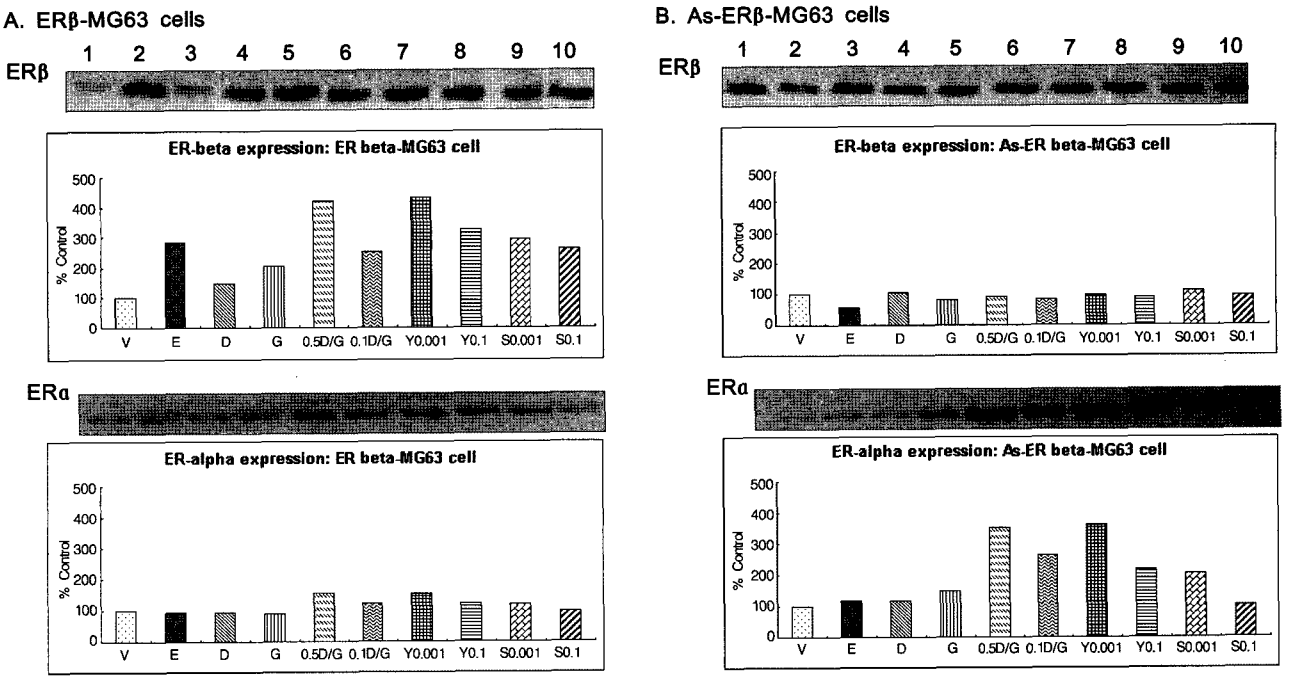

Fig. 2 Representative expression of ERβ and ERα in transfected MG-63 cells.

Protein extracts (10 μg/lane) from pcDNA3-ERβ (ERβ-MG63 cells: A) or pcDNA3-As-ERβ (As-ERβ-MG63 cells: B) transfected MG-63 cells treated with vehicle (DMSO only, lane 1), E2 (10^{-9} M, lane 2), daidzein (10^{-6} M, lane 3), genistein (10^{-9} M, lane 4), genistein+daidzein (0.5×10^{-8} M/each, 0.1×10^{-8} M/each lanes 5-6), Yak-kong (0.001, 0.1 mg/mL, lanes 7-8) or soybean (0.001, 0.1 mg/mL, lanes 9-10) were subjected to SDS-PAGE and immunoblotting with ERβ or ERα specific antibodies. Densitometer analysis: For ERβ or ERα protein levels, the fold induction of each treated cells was normalized first to the corresponding value of actin (not shown) and then calculated to the area value in vehicle treated cells (lane 1).

(Fig. 2). When compared to DMSO (vehicle) treated ERβ -MG63 cells (Fig. 2A, lane 1: 100%), 10^{-9} M estrogen or genistein treatment increased the ERβ expression to 284.2% but 10^{-6} M daidzein treatment increased the ERβ expression only to 145.1%, which was lower than the effects of low concentration of estrogen or genistein treatment on the ERβ expression. Yak-kong and soybean extract treatments were inversely related to the concentration and more significantly increased the ERβ expression up to 432.1%. While the ERβ expression was increased by 0.001 mg/mL Yak-kong extract treatment to 432.1% (Fig. 2A, ERβ: lane 7) it was increased to 291.6% by 0.001 mg/mL soybean extract treatment (Fig. 2A, ERβ: lane 9), and the ERβ expression by 0.001 mg/mL Yak-kong extract treatment was similar to the increase of the ERβ expression by combined standard substances of daidzein and genistein at 0.5×10^{-8} M/each that was corresponding to the concentrations of daidzein and genistein in 0.001 mg/mL Yak-kong extract (Fig. 2A, ERβ: lane 5, 420.8%). The treatment of 10^{-9} M estrogen on As-ERβ-MG63 cells in which the ERβ expression was suppressed more inhibited the ERβ expression and also the degree of ERβ expression was maintained at the level of 80.6%-110.8% even by Yak-kong or soybean extract treatment in addition to daidzein and genistein standard substances (Fig. 2B, ER β: lanes 3-10), and the degree of the ERβ expression by these treatments was similar to that of vehicle (DMSO) only treated As-ERβ -MG63 cells (Fig. 2B, ER β: lane 1). The treatment of 10^{-9} M estrogen and genistein and 10^{-6} M daidzein on ERβ-MG63 cells did not greatly change the ERα expression but the treatment of Yak-kong and soybeam extracts was inversely related to the concentration and increased the ERα expression up to 155.2% (Fig. 2A, ERα: lanes 7-10). The treatment of 10^{-9} M estrogen and genistein and 10^{-6} M daidzein on As-ERβ-MG63 cells increased the ERα expression up to 148.3%, and the 0.001 mg/mL Yak-kong extract treatment even more increased the up to 362.4% and the 0.001 mg/mL soybean extract treatment increased the ERα expression up to 204.2% (Fig. 2A, ERα: lanes 2-4, 6-10), and these maximum increase was more than twice higher than the increase of ERα expression by Yak-kong and soybean extract treatment for ERβ-MG63 cells. The ERβ expression in ERβ-MG63 cells and the ERα expression in ERβ-MG63 and As-ERβ-MG63 cells showed more significant increase effect by Yak-kong treatment than by soybean treatment, and the effect was similar to the

increase effect by combined standard substances of daidzein and genistein at 0.5×10^{-8} M/each that was corresponding to the concentrations of daidzein and genistein in 0.001 mg/mL Yak-kong extract. These results suggest that the changes in the expression of ERβ and ERα by Yak-kong extract treatment in ERβ-MG63 and As-ERβ-MG63 cells are due to the synergic effect of low concentration isoflavones.

3. Changes in the Proliferation of ERβ-MG63/ As-ERβ-MG63 Human Osteoblastic Cells by Yak-kong or Soybean Treatment

Changes in the proliferation of ERβ-MG63 and As-ERβ-MG63 cells by Yak-kong or soybean extract treatment in addition to each standard substance of estrogen, daidzein, and genistein were examined (Table 1). The treatment of 10^{-9} M estrogen and genistein and 10^{-6} M daidzein on ERβ-MG63 cells did not influence the proliferation. Also, in addition to 0.001~0.1 mg/mL Yak-kong and soybean extract treatment, the combined standard substance treatment of daidzein and genistein with the corresponding concentration of 0.001 mg/mL of these extracts did not influence the proliferation. Thus in case of increased ERβ expression, treatment of estrogen, isoflavone standards, Yak-kong or soybean extract did not influence the proliferation of MG-63 osteoblastic cells.

On the other hand, the treatment of 10^{-9} M estrogen and genistein and 10^{-6} M daidzein on As-ERβ-MG63 cells increased the proliferation up to 139.1%. The treatment of 0.001~0.1 mg/mL Yak-kong and soybean extract on As-ERβ-MG63 cells was inversely related with treatment concentration and more significantly increased the proliferation up to 167.8%. The effect of increased proliferation by 0.001 mg/mL Yak-kong extract and soybean extract treatment was similar to the increased proliferation by combined standard substance

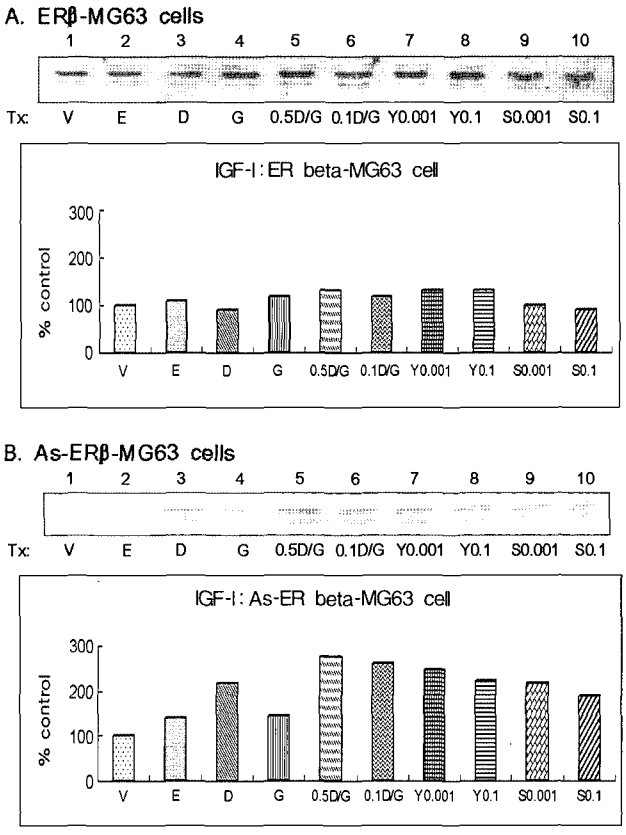

Fig. 3 Representative expression of IGF-I in transfected MG-63 cells

Protein extracts (10 μg/lane) from pcDNA3-ERβ (**ERβ-MG63 cells: A**) or pcDNA3-As-ERβ (**As-ERβ-MG63 cells: B**) transfected MG-63 cells treated with *vehicle (DMSO only, lane 1), E2 (10^{-9} M, lane 2), daidzein (10^{-6} M, lane 3),* genistein (10^{-9} M, lane 4), genistein+daidzein (0.5×10^{-8} M/each, 0.1×10^{-8} M/each lanes 5-6), Yak-kong (0.001, 0.1 mg/mL, lanes 7-8) or soybean (0.001, 0.1 mg/mL, lanes 9-10) were subjected to SDS-PAGE and immunoblotting with IGF-I specific antibody.

Densitometer analysis: For IGF-I protein levels, the fold induction of each treated cells was normalized first to the corresponding value of actin (not shown) and then calculated to the area value in vehicle treated cells (lane 1).

treatment of daidzein and genistein with the corresponding concentrations of each extract. In addition to the significantly increased ERα expression by these treatments in As-ERβ-MG63 cells (Fig. 2B), it is considered that

Table 1. Proliferation of transfected MG-63 cells[1)]

Transfected MG-63 Cells	Treatment[2)]											
	Vehicle (DMSO)	E_2 (10^{-9}M)	D (10^{-6}M)	G (10^{-9}M)	D+G (M)		Yak-kong (mg/mL)			Soybean (mg/mL)		
					0.5×10^{-8}/ each	0.1×10^{-8}/ each	0.001	0.01	0.1	0.001	0.01	0.1
ERβ	100±6.5^{a}	101.3±13.4^{a}	96.7±7.7^{ab}	94.4±9.7^{ab}	101.9±6.2^{a}	100.4±8.2^{a}	100.4±8.2^{a}	96.7±7.3^{ab}	94.8±10.7^{ab}	101.3±10.4^{a}	101.3±13.5^{a}	97.5±6.0^{ab}
As-ERβ	100±4.7^{c}	122.3±5.7^{c}	128.2±8.3^{c}	139.1±7.5^{b}	161.1±7.2^{a}	140.5±11.4^{b}	167.8±4.7^{a}	141.7±3.1^{b}	138.3±6.8^{b}	139.7±5.9^{b}	110.1±5.8^{d}	88.0±8.9^{f}

1) Values are mean±SD (%) from three independent experiments done in 10 samples/treatments. Different letters in the same row indicate significant differences ($P<0.05$).

2) pcDNA3-ERβ (ERβ) or pcDNA3-As-ERβ (As-ERβ) transfected MG-63 cells were plated at 0.5×10^{4} cells/well. After 24 hrs, estrogen (E2:10^{-9} M), daidzein (D:10^{-6}M) genistein (G:10^{-9} M), daidzein+genistein (D+G: 0.5×10^{-8} M/each, 0.1×10^{-8} M/each), yak-kong (0.001, 0.01 and 0.1 mg/mL) or soybean (0.001, 0.01 and 0.1 mg/mL) were added at an indicated concentration. After 5 days, MTS solution was added and absorbance was measured at 490 nm.

the increased effect of the proliferation by Yak-kong or soybean extract treatment in addition to standard substances of estrogen, daidzein and genistein in As-ERβ-MG63 cells is mediated by ERα.

4. Changes in the Expression of IGF-I by Yak-kong or Soybean Treatment in ERβ-MG63/As-ERβ-MG63 Human Osteoblastic Cells

Among several factors that induce the proliferation of MG-63 human osteoblastic cells, IGF-I has been reported as a major proliferation-inducing factor for MG-63 cells by estrogen, isoflavone, and Yak-kong and soybean extracts in the previous studies.[15,16] Thus, changes in the expression of ERβ and the treatment effect by Yak-kong or soybean extract in addition to standard substances of estrogen, daidzein and genistein on the expression of IGF-I in MG-63 cells were examined (Fig. 3). The treatment of 10^{-9} M estrogen and genistein and 10^{-6} M daidzein on ERβ-MG63 cells increased the IGF-I expression up to 121.0% (Fig. 3A: lanes 3-5). Yak-kong and soybean extracts, and the combined standard substance treatment of daidzein and genistein with the corresponding concentration of 0.001 mg/mL of these extracts slightly increased the expression of IGF-I up to 130.3% (Fig. 3A: lanes 6-10), but this was not a great increase when compared to single standard substance treatment of estrogen, daidzein and genistein.

The treatment of 10^{-9} M estrogen and genistein in As-ERβ-MG63 cells increased the IGF-I expression up to 216.4% and Yak-kong and soybean extract treatments were inversely related to the concentration and more significantly increased the IGF-I expression. The expression of IGF-I was more increased by 0.001 mg/mL Yak-kong extract than by 0.001 mg/mL soybean extract (Fig. 3B: lane 7, 247.2%; lane 8, 222.1 %), which was also similar to the increased IGF-I expression by combined standard substances of daidzein and genistein at 0.5×10^{-8} M/each that was corresponding to the concentrations of daidzein and genistein in 0.001 mg/mL Yak-kong extract (Fig. 3B: lane 5; 276.4%). Results in Fig. 3 showed that the treatment of Yak-kong or soybean extract in addition to standard substances of estrogen, daidzein and genistein on ERβ-MG63 cells slightly increased the expression of IGF-I, a proliferation-inducing factor, but were considered as not sufficient to increase the proliferation of MG-63 cells (Table 1) along with insufficient increase of ERα expression (Fig. 2A). On the other hand, the treatment of Yak-kong or soybean extract in addition to standard substances of estrogen, daidzein and genistein on As-ERβ-MG63 cells significantly increased not only the expression of IGF-I (Fig. 3B) but also the expression and proliferation of ERα (Fig. 2: Table 1), which suggests that the expression of ERα rather than ERβ was significantly increased in MG-63 osteoblastic cells which proliferation was increased by the treatment of Yak-kong or soybean extract in addition to standard substances of estrogen, daidzein and genistein,[15,16] and when the ERα expression is increased by the transfection of pcDNA3-ERα plasmid (ERα-MG63 cell), the increased effect of proliferation by additional treatment of Yak-kong or soybean extract was more significantly increased in the previous study,[17] and that the increased proliferation effect of isoflavones contained in Yak-kong or soybean extract in addition to standard substances of estrogen, daidzein and genistein on MG-63 osteoblastic cells is mediated by ERα receptors. Also, results in Fig. 3 and Table 1 suggest that ERβ might

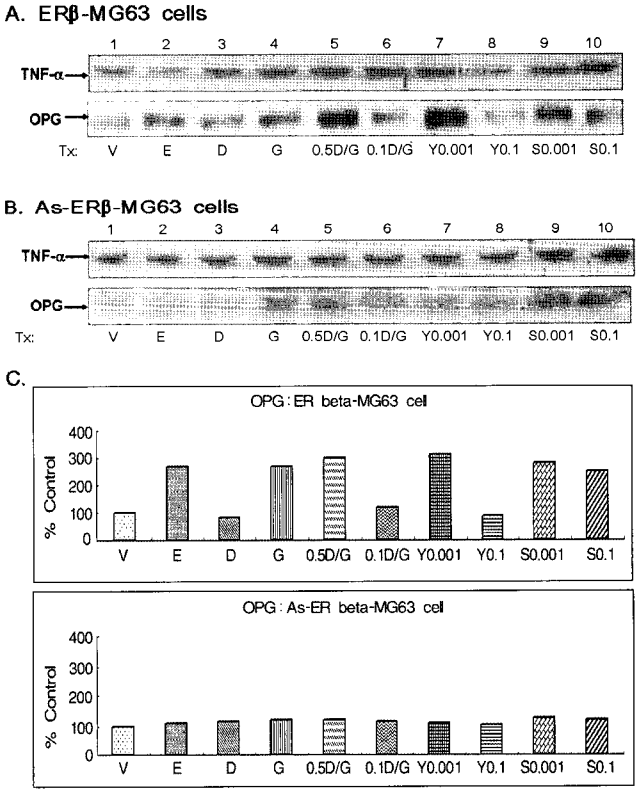

Fig. 4 Representative expression of OPG and TNFα in transfected MG-63 cells

Protein extracts (10 μg/lane) from pcDNA3-ERβ (**ERβ-MG63 cells: A**) or pcDNA3-As-ERβ (**As-ERβ-MG63 cells: B**) transfected MG-63 cells treated with vehicle (DMSO only, lane 1), E2 (10^{-9} M, lane 2), daidzein (10^{-6} M, lane 3), genistein (10^{-9} M, lane 4), genistein+daidzein (0.5×10^{-8} M/each, 0.1×10^{-8} M/each lanes 5-6), Yak-kong (0.001, 0.1 mg/mL, lanes 7-8) or soybean (0.001, 0.1 mg/mL, lanes 9-10) were subjected to SDS-PAGE and immunoblotting with OPG or TNFα specific antibody. **C.** The signal intensities of OPG in A and B were quantified and the integrated areas were normalized, first to the corresponding value of actin (not shown) and then calculated to the area value in vehicle treated cells (lane 1).

have another role in bone metabolism other than inducing the proliferation of osteoblasts.

5. Changes in the Expression of Differentiation-inducing Factor in ERβ-MG63/As-ERβ-MG63 Human Osteoblastic Cells by Yak-kong or Soybean Treatment

MG-63 osteoblastic cells synthesize and secrete local factors including several proliferation-inducing factors, Tumor Necrosis Factor-α (TNF-α), Interleukin -1 (IL-1), and Interleukin-6 (IL-6) that inhibit the activity of osteoblasts and increase the differentiation and activity of osteoclasts. These are factors promoting bone resorption and estrogen and isoflavone have been known to inhibit the expression of these factors and indirectly inhibit bone resorption.[23] Recently, Osteoprotegerin (OPG) was found as a local factor secreted from osteoblasts and its expression and secretion have been known to be increased by estrogen treatment to strongly inhibit the osteoclast differentiation, which is different from TNF-α.[24] The effects of Yak-kong or soybean extract in addition to standard substances of estrogen, daidzein and genistein on the expression of TNF-α and OPG in ERβ-MG63 and As-ERβ-MG63 cells were examined to find out the roles of ERβ in bone metabolism other than inducing the proliferation of osteoblasts (Fig. 4). TNF-α was expressed in DMSO treated ERβ-MG63 and As-ERβ-MG63 cells but the change in the expression by Yak-kong or soybean extract in addition to standard substances of estrogen, daidzein and genistein was slight at 93.5~105.2% level (Fig. 4A and B), and thus it is considered that the expression of TNF-α is not influenced by changes in ERβ expression and estrogen and isoflavone treatments.

The treatment of 10^{-9} M estrogen and genistein on ERβ -MG63 cells increased the expression of OPG (Fig. 4A and C: lane 2: 266%, lane 4: 270%) but the treatment of 10^{-6} M daidzein decreased the expression of OPG. The expression of OPG by 0.001 mg/mL Yak-kong extract was more significant than by 0.001 mg/mL soybean extract (Fig. 4A and C: lane 7, 305%, lane 9, 268.2%), which was similar to the increased OPG expression by combined standard substances of daidzein and genistein at 0.5×10^{-8} M/each that was corresponding to the concentrations of daidzein and genistein in 0.001 mg/mL Yak-kong extract (Fig. 4A and C: lane 5; 297.8%). On the other hand, the treatment of 10^{-9} M estrogen and genistein and 10^{-6} M daidzein on As-ERβ-MG63 cells did not greatly affect the OPG expression. Also, in addition to 0.001~0.1 mg/mL Yak-kong extract treatment, the combined standard substance treatment of daidzein and genistein with the corresponding concentration of 0.001 mg/mL of these extracts did not influence the OPG expression. The treatment of 0.001~0.1 mg/mL soybean extract on As-ERβ-MG63 cells slightly increased the OPG expression up to 110.8% (Fig. 4B and C: lanes 9-10) but not greatly increased compared to the treatment by single standard substance of estrogen, daidzein and genistein. Results in Fig. 4 suggest that the significant increase in the expression of OPG, which is secreted from osteoblasts and inhibits the osteoclast differentiation, is selectively increased by synergistic effect of increased ERβ expression and low levels of isoflavone contained in Yak-kong extract.

SUMMARY & CONCLUSION

This study was performed to produce MG-63 human osteoblastic cell line with increased ERβ expression (ER β-MG63 cell line) and MG-63 human osteoblastic cell line with extremely suppressed ERβ expression (As-ERβ -MG63 cell line) with the method of molecular biology, and to measure the changes in the expression of differentiation factors that are secreted from osteoblasts and influence the osteoclast differentiation in addition to the proliferation and expression of estrogen receptors and IGF-I after the treatment of Yak-kong or soybean extract to each cell line, and to examine the role of ERβ for the expression of differentiation factors by Yak-kong or soybean treatment in MG-63 osteoblastic cells. The results are summarized as below.

1) The ERβ expression was increased by gene transfection of pcDNA3-ERβ plasmid (ERβ-MG63 cells) and more significantly increased by the treatment of Yak-kong or soybean extract in addition to standard substances of estrogen, daidzein, and genistein on ERβ-MG 63 cells. Also, these treatments slightly increased the expression of ERα and IGF-1, but did not influence the proliferation.
2) The treatment of Yak-kong or soybean extract in addition to standard substances of estrogen, daidzein, and genistein on ERβ-MG 63 cells with suppressed ERβ expression did not influence the expression of ERβ. On the other hand, these treatments significantly increased the expression of ERα and IGF-1 in addition to cell proliferation, among which the increase of proliferation was the highest in the treatment by Yak-kong extract and similar to the increased proliferation effect by the treatment of combined standard substances of daidzein and

genistein with the concentration corresponding to this extract.

3) Changes in the expression of TNF-α and OPG by Yak-kong and soybean extract treatment in ERβ-MG 63 cells and As-ERβ-MG 63 cells were examined and the expression of TNF-α was not changed by the treatment of Yak-kong or soybean extract in addition to standard substances of estrogen, daidzein, and genistein in both ERβ-MG 63 and As-ERβ-MG 63 cells. On the other hand, the treatment of Yak-kong or soybean extract in addition to standard substances of estrogen, daidzein, and genistein on ERβ-MG 63 cells increased the OPG expression, among which Yak-kong extract showed the highest increase effect on OPG expression. This was similar to the increased OPG expression by the treatment of combined standard substances of daidzein and genistein with corresponding concentration to this extract. This significant increase in the expression of OPG by Yak-kong extract was not observed in ERβ-MG 63 cells in which the ERβ expression was suppressed.

The above results suggest that the proliferation of MG-63 human osteoblastic cells and the increased expression of IGF-I by Yak-kong or soybean extract treatment are induced by ERα receptor rather than ERβ receptor, while the ERβ receptor induces the increased selective expression of OPG by Yak-kong treatment in MG-63 human osteoblastic cells. It is considered that Yak-kong extract treatment, which is low concentration isoflavone mixture, on MG-63 human osteoblastic cells indirectly inhibits the differentiation of osteoclasts by inducing the increased selective expression of OPG through the binding of isoflavone and ERβ.

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