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Effects of Genistein on expression of bone markers during MC3T3-E1 osteoblastic cell differentiation

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Abstract

In this *in vitro* study, the hypothesis that the beneficial effects of dietary genistein on bone are through the modulation of the bone marker synthesis by osteoblastic MC3T3-E1 cells was tested, and the possible roles of estrogen receptors in the actions of genistein on osteoblastic cells were also examined. Interleukin-6 production was decreased 40% to 60% in osteoblastic cells treated with genistein from either day 8–16 or day 12–16, at dietarily achievable concentrations (10^{-10} to 10^{-8} M) ($P<0.05$). The mRNA expression of osteoprotegerin increased about 140% in cells treated from with genistein day 4–8 at a concentration of 10^{-8} M ($P<0.05$). The ratio of estrogen receptor-α to β expression increased 10-fold from day 0 to 12 of culture ($P<0.05$). Correlating with this time-dependent variation in estrogen receptor expression, treatments of 17β-estradiol and genistein had opposite dose patterns on the ratio of estrogen receptor-α to β expression following treatment from day 4 to 6 compared to from day 0 to 2. The addition of ICI-182,780, an estrogen receptor blocker, reduced the inhibitory effect of genistein on IL-6 production by 30-50%. In summary, these findings suggest that the beneficial skeletal effects of genistein, at dietarily achievable levels, appear to be mediated, at least in part, by interleukin-6 and osteoprotegerin, and estrogen receptors play important roles in the inhibition of interleukin-6 synthesis by genistein in osteoblastic MC3T3-E1 cells.

Keywords: Genistein; Estrogen receptor-α and β; Osteoblast; Interleukin-6; Osteoprotegerin

1. Introduction

Because postmenopausal osteoporosis results from endogenous estrogen deficiency, the role of estrogens and estrogen-like molecules in regulating activities of bone cells is central to understanding the etiology and treatment of this bone disease. Although estrogen replacement therapy (ERT) has been a widely used approach for preventing postmenopausal osteoporosis, ERT not only increases the risk of breast cancer but it also has other undesirable side-effects [1].

In the past few years, natural estrogen-like molecules, namely soy isoflavones, have attracted attention for their potential roles in osteoporosis prevention [2]. For example, young ovariectomized (OVX) rats or mice have demonstrated consistent improvement in the retention of bone after ovariectomy when treated with isoflavone-containing soy or pure isoflavones [3–6]. In human studies, dietary supplementation with isoflavone-enriched soy products improved vertebral bone mass of both estrogen-deprived peri- and post-menopausal women [7,8], but not of women with normal menstrual cycles [9].

The cellular mechanisms through which dietary isoflavones may enhance the retention of bone remain unclear. Isoflavones have been demonstrated both to act via classical estrogen receptor-mediated signaling and to function as tyrosine kinase inhibitors [10]. The discovery by Kuiper *et al* of the β isoform of the estrogen receptor (ER) has raised a number of questions regarding the respective physiological roles that these two receptors, ER-α and ER-β, play in estrogen-responsive tissues [11].

Besides the classical bone formation markers, such as alkaline phosphatase (ALP) and osteocalcin, several other products synthesized by osteoblasts, including interleukin-6

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(IL-6), osteoprotegerin (OPG), and receptor activator of NF-κB ligand (RANKL), may also be regulated by genistein. These three cytokine products have been demonstrated to influence osteoclast differentiation and activation [12–14]. The effects of genistein, at dietarily achievable concentrations, on these cytokines, however, have not been tested in osteoblasts.

The purpose of this *in vitro* study was to characterize the actions of genistein on bone marker synthesis during cell differentiation of MC3T3-E1 cells, an osteoblast-like cell line derived from newborn mouse calvariae. Based on the changes of osteoblastic phenotype during MC3T3-E1 cell differentiation, the possible roles of ERs in osteoblastic cells following genistein treatments were also examined.

2. Methods and materials

2.1. Materials

MC3T3-E1 cells were obtained from Dr. Quarles (Duke Medical Center, Durham, NC). Quantitative RT-PCR primers were synthesized at the University of North Carolina Lineberger Comprehensive Cancer Center Nucleic Acid Core Facility. IL-6 ELISA kit was purchased from Pharmingen Inc (San Diego, CA). Cell culture media (α-MEM with or without phenol red), antibiotics, and TRIzol reagents were purchased from Gibco-BRL (Rockville, MD). Fetal Bovine Serum (charcoal/dextran treated) was purchased from Hyclone (Logan, Utah). If not mentioned specifically, all other reagents were purchased from Sigma (St. Louis, MO).

2.2. Cell culture

MC3T3-E1 cells were cultured in phenol red free α-EM supplemented with 10% charcoal-stripped FBS, 100 U/ml penicillin and 10 mg/ml streptomycin at 37°C in 5% CO_2-95% air. Cells were subcultured at twice weekly intervals with 0.01% pronase. For induction of differentiated phenotype, culture medium was also supplemented with 25 μg/ml of ascorbate and 1 μg/ml β-glycerophosphate. For treatment, 17β-estradiol or genistein were added with fresh medium on a scheduled feeding day. Cells and culture medium were collected for isolation of total RNA, IL-6 ELISA, and alkaline phosphatase assay, etc.

2.3. Total RNA isolation

Total cellular RNA was isolated using TRIzol reagent, according to protocols provided by the manufacturer. In brief, after homogenization with TRIzol, 0.1 ml chloroform was added for each 0.5 ml of TRIzol usage, and the sample was centrifuged at less than 12,000 g for 15 min. Then, the aqueous phase was transferred into a new tube and 0.25 ml of isopropanol was added. After centrifugation at 12,000 g for 10 min, the RNA was precipitated as a white pellet at the bottom of the tube. The RNA pellets in 75% ethanol were stored at –20°C until analysis.

2.4. RT-PCR (real time)

Approximately 800 ng of total RNA was used as a template to synthesize first strand complementary DNA (cDNA) with specific reverse primers for osteocalcin (OCN), collagen type I (COL I), IL-6 OPG, RANKL, and GAPDH genes, using reverse transcription (RT). Then, 2 μl of the cDNA mixture was used in a real time PCR reaction (20 μl total volume) performed with Smart Cycle TD (Cepheid, Sunnyvale, CA) following methods recommended by the manufacturer. Optimal conditions were defined as: Step 1, 95°C for 10 min; Step 2, 94°C for 15 sec, 62°C for 10 sec, 72°C for 15 sec; repeated for 40 cycles. The products were electrophoresed on 1% agarose gels. The relative mRNA expressions adjusted with GAPDH and the ratio of ER-α to ER-β was using as the endpoints in this study. In addition, PCR products had been purified and sent for sequence analysis using ABI100 system (Model 377) to confirm the correct amplification of targeted genes.

2.5. ELISA for IL-6

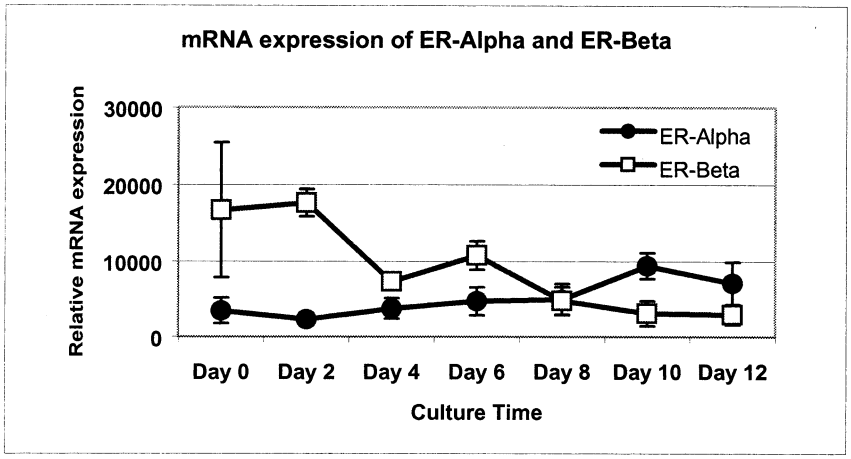
IL-6 was measured using a dual antibody ELISA kit purchased from Pharmingen, Inc. In brief, pipette 100 μl of standard, sample, or control into 96-well plate pre-coated with mouse IL-6 antibody. After incubation for two hours, plate was washed for 4 times. Then, 100 μL detecting solution with biotinylated affinity IL-6 antibody was added to each well. Plates then were covered with plate sealer and were incubated for 1 hr at room temperature. After washing plate thoroughly, 100 μL substrate solution was added into each well to develop the color reaction. After 30 min, stop solution was added. IL-6 concentrations were quantified using absorbency at 450 nm within 30 min on a microplate reader (Bio-Rad Laboratory, Hercules, CA). The IL-6 measurements were adjusted for DNA content.

2.6. Assay for DNA

DNA was measured by using the method of Labarca and Paigen [15]. In brief, the cell samples to be analyzed were homogenized in P-S buffer and sonicated briefly. Then 1 ml of working Hoechst dye solution was added to a standard or sample tube and mixed gently. The samples were measured using a fluorescence spectrophotometer (F-2000, Hitachi Instruments, Inc., Danbury, CT) at excitation 358 nm and emission 450 nm.

2.7. Assay for alkaline phosphatase activities

ALP was measured by using a modification of the King-Kind method [16]. In brief, the cell samples to be analyzed



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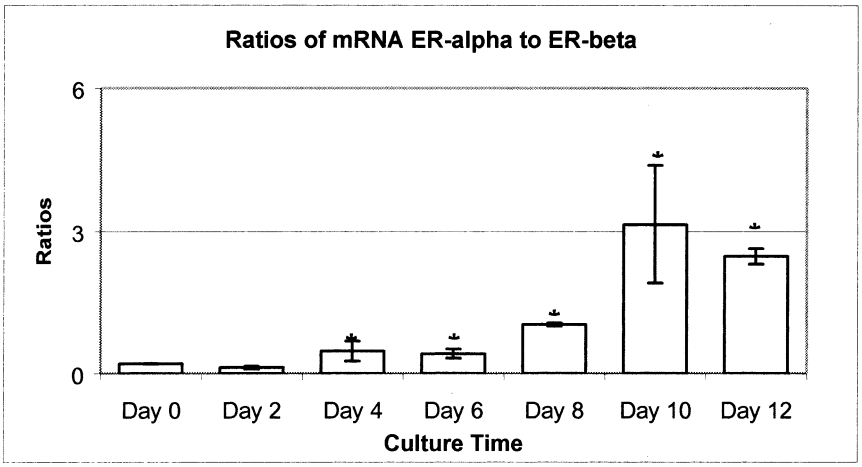

B

Fig. 1. Time course (days 0-12) of changes in estrogen receptor α and β mRNA expression in MC3T3-E1 cells.MC3T3-E1 cells were cultured for up to 12 days, and cell samples were collected on days 2, 4, 6, 8, 10, and 12. Measurements of ER mRNA expression RT-PCR (real time) was performed with specific forward and reverse primers for ER α & β. In addition, the ratios of mRNA ER α to β were also calculated. **(A)** mRNA expression of ER α and ER β; **(B)** Ratio of mRNA ER α to ER β *(*: vs. Day 0, $p < 0.05$, Duncan's Multiple Range Test).* Mean ± SD.

were homogenized in P-S buffer and sonicated briefly. Then 0.25 ml alkaline buffer solution and 0.25 ml stock substrate solution were added, and test tubes were placed in a 37°C water bath to equilibrate. 0.25 ml of homogenized cell samples was then pipeted into the test tubes and mix gently. After exactly 15 min in the water bath, 5 ml of 0.05N NaOH was added to each tube and mixed by inversion. Absorbance of TEST vs. BLANK as reference was read at 420 nm using a spectrophotometer (U-2000, Hitachi Instruments, Inc., Danbury, CT). Alkaline phosphatase units were determined by comparison to the standard curve.

2.8. Statistics

Duncan's Multiple Range Test was employed using SAS software 6.0 (SAS Institute, Cary, NC). A value of $P < 0.05$ was considered significant and results are presented as the mean ± standard deviation (SD).

3. Results

3.1. Development of osteoblastic phenotypes during MC3T3-E1 cell differentiation

The expression of both ER-α & -β in MC3T3-EI during cell differentiation was examined. MC3T3-E1 cells were cultured for up to 12 days, and samples were collected on days 0, 2, 4, 6, 8, 10, and 12. Real time RT-PCR was performed with specific forward and reverse primers for ER-α & -β, and products were electrophoresed on 1% agarose gels. The predicted product sizes of 431 bp and 355 bp for ER-α and ER-β, respectively, were obtained. Real time PCR standard curves for ER-α, ER-β and other target genes, such as GAPDH, OPG, and RANKL, in this study were linear over four concentrations. The linear correlations (r) between the number of target gene copies and the threshold cycles (C_T) were ≥ 0.99 in each case. For ER mRNA

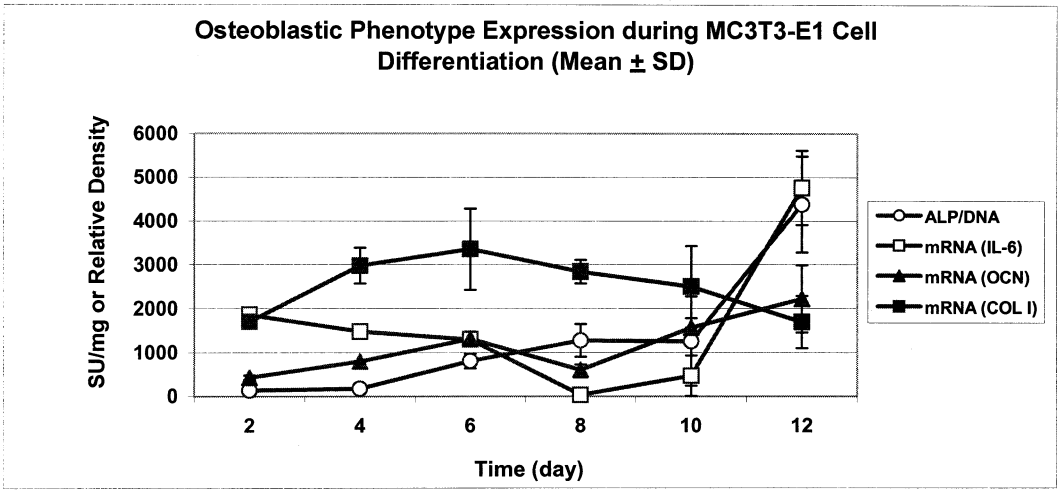

Fig. 2. Time Course (days 0-12) of changes in alkaline phosphatase (ALP), osteocalcin (OCN), collagen type I (COL I), and interleukin-6 (IL-6) expression in MC3T3-E1 cells.MC3T3-E1 cells were cultured for up to 12 days, and cell samples were collected on days 2, 4, 6, 8, 10, and 12. ALP assay, IL-6 ELISA and RT-PCR was performed with specific forward and reverse primers for OCN, COL I and IL-6. No treatments were given. *(*: vs. Day 0, $p < 0.05$, Duncan's Multiple Range Test).*

expressions, although no significant change in ER-α mRNA was found, ER-β mRNA decreased significantly from the immature period (day 0 to 2) to normal differentiation period (day 10-12) ($P<0.05$) (Fig. 1A). As a result, the ratio of ER-α to ER-β expression increased about 3-fold from day 0 to day 6, and 10-fold from day 0 to 12 ($P<0.05$) (Figure 1B). In addition, the expression of other bone markers during MC3T3-E1 cell differentiation was also examined. ALP, OCN and IL-6, increased, whereas COL I decreased during MC3T3-E1 cell differentiation after reaching the peak at day 6. The ALP activities exhibited an 80-fold significant increase from the immature period (day 2) to the normal differentiation period (day 12), and both IL-6 production and mRNA expression exhibited significant increases after the cells became differentiated (Fig. 2). These findings indicate that the mouse MC3T3-E1 cell line undergoes a normal developmental sequence of osteoblast differentiation in association with the formation of bone markers.

3.2. Actions of genistein on cell differentiation of MC3T3-E1

3.2.1. Effects of genistein on bone markers expressed by MC3T3-E1 cells during differentiation

After pre-culturing, 17β-estradiol (E_2) or genistein (GEN) was added into the culture media at day 8 or day 12. After harvesting on day 16, the cells and media were collected for DNA and IL-6 assay. As shown in Fig. 3A, significant decreases (about 40-60%) in IL-6 production were found between vehicle (control) and all cell groups treated with 17β-estradiol or genistein. The effects of genistein on IL-6 synthesis were slight dose dependent. In addition, the cells treated with genistein from day 8 to 16 (8-day treatment) had less IL-6 production than those treated from day 12 to 16 (4-day treatment), therefore, the inhibitory effects of genistein on IL-6 production were also time dependent. Additional ICI 182,780 (10^{-6}M) abolished about 30-50% of inhibitory effects on IL-6 productions by estrogen and genistein (Fig. 3B).

After incubating for four days, MC3T3-E1 cells were treated with 17β-estradiol or genistein at a physiological concentration (10^{-10} to 10^{-8} M) for from day 5 to day 8 (96 hr). Cell samples were collected for total RNA isolation. Quantitative real time RT-PCR was performed with gene-specific primers, and the predicted product sizes of 360 bp, 466 bp and 631 bp for OPG, RANKL and GAPDH, respectively, were obtained. In out study, no RANKL mRNA signal was detectable in vehicle (control), E2, or GEN treated cells (Fig. 4A). The OPG mRNA levels (after adjustment for GAPDH mRNA levels) were found to increase about 180%, 140%, in the osteoblastic cells treated with E_2 (10^{-8} M) , or GEN (10^{-8} M), respectively, compared with vehicle (control) (Figure 4B and 4C), but no changes were found in the cells treated with genistein at the concentration of 10^{-8} M.

The effects of 17β-estradiol and genistein on the ALP activities and mRNA expression of OCN in MC3T3-E1 cell line during cell differentiation were examined. For treatment, 17β- estradiol or genistein were added with fresh medium on either day 8 or day 12. After harvesting on day 16, the cells were collected for ALP and OCN assay. No statistically significant differences in ALP production and OCN mRNA expression were found between control and cells treated with genistein (Data not shown).

3.2.2. Effects of 17β-estradial and genistein on estrogen receptors α and β expression

Based on the data on the changes of ER expression (the ratio of ER-α to ER-β expression increased about 3-fold from day 0 to day 6) during MC3T3-E1 cell differentiation

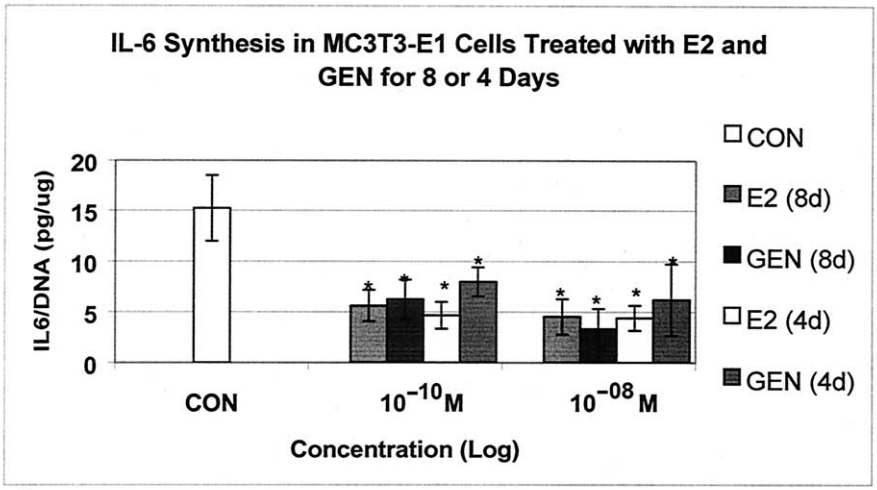

Fig. 3. Effects of genistein on IL-6 production.MC3T3-E1 cells were seeded in 12-well plates at a concentration of 3 x 10^4 cells/cm^2. **(A)** After pre-culturing, 17β-estradiol (E_2) or genistein (GEN) were added into the culture medium at day 8 or day 12. At the end of each experiment, the MC3T3 cells were harvested on day 16. **(B)** 17β-estradiol (E_2) or genistein (GEN) in combination with antiestrogen, ICI-182,780, were added into the culture medium at the same schedule described as above, the MC3T3 cells were harvested on day 16. Cells samples and medium were collected for IL-6 ELISA and DNA assay. IL-6 production was adjusted for DNA contents *(*: vs. CON, $p < 0.05$, Duncan's Multiple Range Test).* Mean ± SD.

(Fig. 1), 17β-estradiol or genistein was added with fresh medium on either day 0 or day 4, and was removed 48 hr later. On day 12, the cells were collected for ER mRNA expression quantification by real time RT-PCR. As shown in Fig. 5, both 17β-estradiol and genistein decreased the ratio of mRNA expression of ER-α to ER-β except the cells treated with genistein from day 0-2 ($P<0.05$). Also, the data showed clearly the reversed time-response patterns for the treatments with 17β-estradiol or genistein.

4. Discussion

In this *in vitro* study, genistein treatment (10^{-10} to 10^{-8} M) significantly decreased IL-6 production, in a time-dependent manner, by about 40-60% (Fig. 3A). However, genistein, at a concentration of 10^{-8} M, also increased the mRNA expressions of OPG about 140% (Fig. 4). During MC3T3-E1 cell differentiation, ER-α mRNA expression was kept more constent than the expression of ER-β mRNA (Fig. 1). Treatments of 17β-estradial and genistein showed opposite effects on the ratios of ERα to β mRNA expression following treatments at the early stages of cell differentiation (Fig. 5). The addition of ICI-182,780, an ER blocker, abolished 30-50% of the inhibitory effect of genistein on IL-6 production (Fig. 3B). The 'physiological' concentrations of genistein used in this study could be readily achievable by the typical dietary intake of isoflavones by Japanese adults [17].

Genistein, in the present study, had relatively little effect

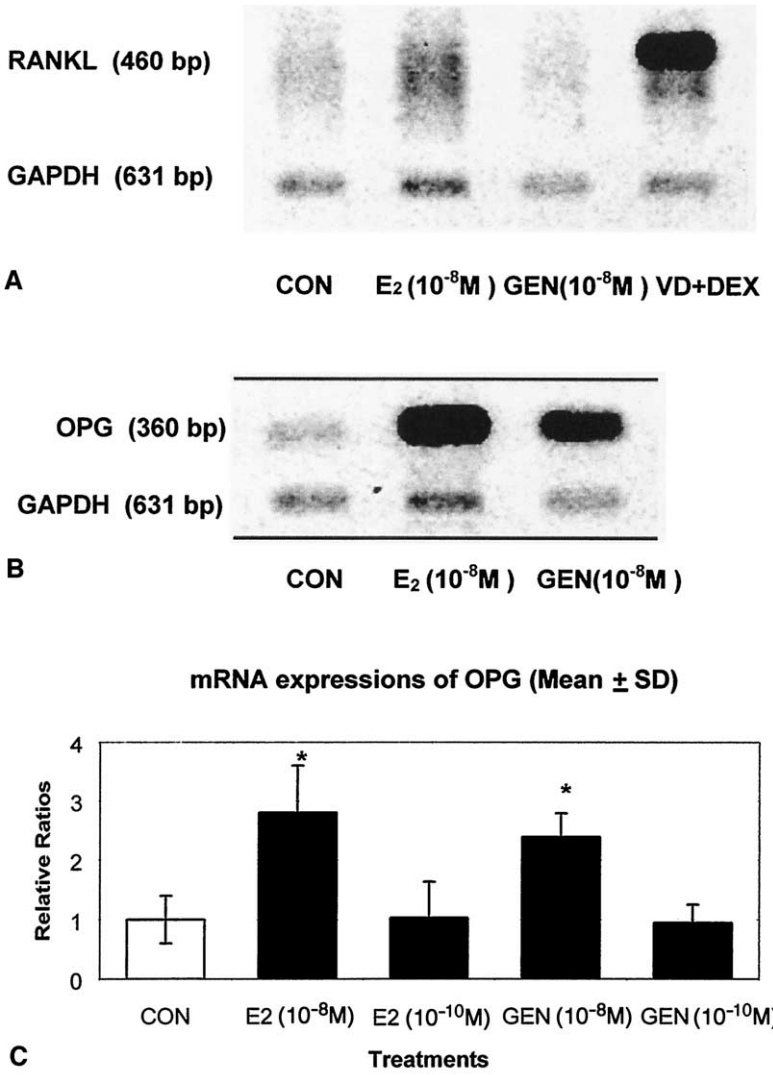

Fig. 4. Effects of genistein on mRNA expression of OPG and RANKLMC3T3-E1 cells were seeded in 12-well plates at a concentration of 3 x 10^4 cells/cm^2. After pre-culturing for 4 days, MC3T3-E1 cells were treated with 17β-estradiol (E_2), or genistein (GEN), were added into the culture medium for the additional culture for another 4 days. The MC3T3-E1 cells were harvested on day 8. Cells samples were collected for RNA isolation. Real time RT-PCR was performed to assay mRNA expression of OPG and RANKL, which was adjusted for GAPDH. (**A**) mRNA expression of RANKL. (**B**) mRNA expression of OPG. (**C**) Quantitative data for mRNA expression of OPG. *(Data expressed as Mean ± SD, *: vs. CON, $P < 0.05$, Duncan's Multiple Range Test).*

on the production of bone formation markers by MC3T3-E1 cells. Little change was found in ALP and OCN production after 16 days of culture. In two previous studies using MC3T3-E1 cells by Sugimoto and Yamaguchi [18,19], genistein and daidzein were shown to elevate alkaline phosphatase activity, but this bone marker was evaluated at an earlier cell proliferation stage (day 2) than we used.

Reports of the effects of isoflavones on osteoblastic phenotype development are limited [17,20]. Most studies of osteoblasts used genistein at the pharmacological range ($\sim 10^{-5}$ to 10^{-3} M) to investigate the roles of secondary messengers in osteoblastic cell mitogenesis [21,22]. At high doses ($\sim 10^{-5}$ to 10^{-3} M), genistein acts mainly as a tyrosine kinase inhibitor, and thereby inhibits cell growth, arrests cell cycle progression at G2-M, and induces apoptosis [23]. At lower concentrations (10^{-10} to 10^{-6} M), which are dietarily achievable levels, genistein appears to function more as a weak estrogenic agonist via ERs [3,24]. Similar independent effects of both 17β-estradiol and genistein were found on IL-6 synthesis, and on OPG and RANKL mRNA expression (Fig. 3 and 4).

Recent reports indicate that genistein may differentially bind to ER-α and ER-β receptors, acting as a partial agonist in ER-β [25]. ER-α and ER-β have been found to distribute differently during osteoblastic cell differentiation [26–28], and estrogen-like molecules, including genistein, have also been found to have different binding affinities to ER-α and ER-β [28]. This finding helps explain the different effects between 17β-estradiol and genistein on the ratio of mRNA expression of ER-α to β (Fig. 5). In previous experiments, we have clearly shown that the regulation of IL-6 and OPG by isoflavones is ER-dependent in the cell mode (hFOB/

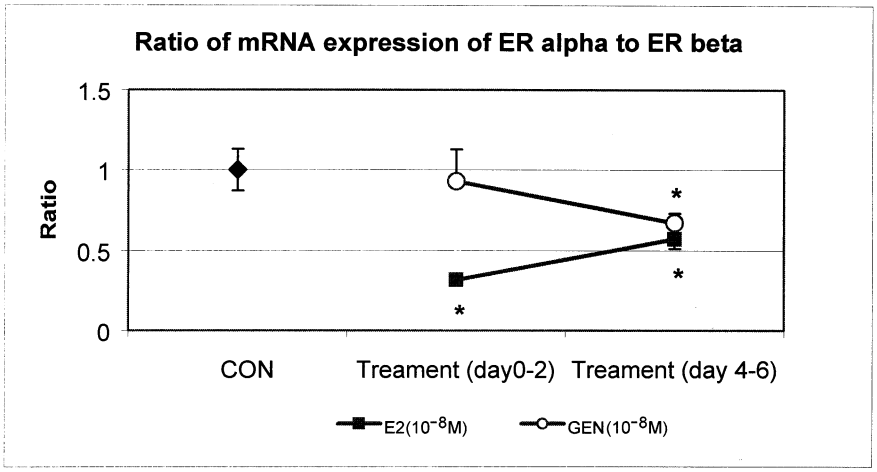

Fig. 5. Effects of genistein (10^{-8} M) on ratio of mRNA expression of ER-α to ER-β. MC3T3-E1 cells were seeded in 12-well plates at a concentration of 3 x 10^4 cells/cm^2. MC3T3-E1 cells were treated with 17β-estradiol or genistein from day 0 to day 2 or from day 4 to day 6. On day 12, the cells were collected for ER mRNA expression assay by real time RT-PCR. *(Data expressed as Mean ± SD, *: vs. CON, P <0.05, Duncan's Multiple Range Test).*

ER9) with high ER presented [29]. However, the mechanism for different effects between genistein on IL-6 in relation to the ratio of mRNA expression of ER-α to β (Fig. 4 and 5) is not clear. The reason for this weak relationship between ER expression and genistein treatment may be related to the very low ER level in the current cell model (MC3T3-E1).

In previous experiments, we have clearly shown that the regulation of IL-6 and OPG by isoflavones is ER-dependent in the cell model (hFOB/ER9) with high ER presented [29]. In comparing with MC3T3-E1 cells, hFOB cells are transforming osteoblastic cell lines, and they are immortalized with temperature-sensitive SV40 large T antigen, which helps control cells at proliferation and differentiation stages. As a non-transforming cell line, MC3T3-E1 cells used in this study are derived from newborn mouse calvariae, and they display osteoblast-like characteristics. They also undergo a normal developmental sequence of osteoblast differentiation in association with the expression of bone markers [30,31]. The possible differences between non-transforming and transforming cell lines need to be considered when interpreting data from studies using these cell lines. Both cell culture systems have advantages and disadvantages: in transforming cell lines, the osteoblastic phenotypes, like proliferation and differentiation, are well controlled. However, one major problem of transforming osteoblastic cell culture systems is that some genetic factors, such as the introduction of exogeneous gene, may have been modified. In these studies, we tried to test the effects of dietary genistein by using a non-transforming cell culture system.

Published evidence supports the concept that estrogen inhibits IL-6 gene transcriptional activation through an ER-dependent mechanism in murine bone marrow stromal cells [32,33]. A previous report also showed that estrogen-regulated IL-6 synthesis was positively associated with the levels of ER expression in osteoblastic cells [34]. In our study, IL-6 production by MC3T3-E1 cells was inhibited 40-60% by both β-estradiol and genistein (Fig. 3A). After adding the antiestrogen, ICI 182,780, the inhibitory effects on IL-6 synthesis by estrogen and genistein were reduced (Fig. 3B). As mentioned previously, at high doses ($\sim 10^{-5}$ to 10^{-3} M), genistein acted mainly as a tyrosine kinase inhibitor, but at physiological concentrations (10^{-10} to 10^{-6} M), which are close to the dietarily achievable levels, ER-dependent pathways, may become dominant.

In summary, the data described in this report suggest that genistein, a common isoflavone derived from soybeans, exerts its effects on osteoblast-like cells at dietarily achievable concentrations, and the beneficial effects of genistein may be partly related to inhibition of osteoclastogenesis as mediated by cytokine production in osteoblasts. This conclusion is supported by the finding that estrogen loss increases the activation of osteoclasts [12]. In addition, inhibition of IL-6 production by genistein is mediated, in a major way, through estrogen receptors and DNA expression rather than by a non-genomic pathway involving tyrosine kinase or other cytosolic enzymes.

Acknowledgments

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