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Daidzein enhances osteoblast growth that may be mediated by increased bone morphogenetic protein (BMP) production

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Abstract

Daidzein, a natural isoflavonoid found in *Leguminosae*, has received increasing attention because of its possible role in the prevention of osteoporosis. In the present investigation, primary osteoblastic cells isolated from newborn Wistar rats were used to investigate the effect of this isoflavonoid on osteoblasts. Daidzein (2–50 μM) increased the viability ($P < 0.05$) of osteoblasts by about 1.4-fold. In addition, daidzein (2–100 μM) increased the alkaline phosphatase activity and osteocalcin synthesis ($P < 0.05$) of osteoblasts by about 1.4- and 2.0-fold, respectively. Alkaline phosphatase and osteocalcin are phenotypic markers for early-stage differentiated osteoblasts and terminally differentiated osteoblasts, respectively. Our results indicated that daidzein stimulated osteoblast differentiation at various stages (from osteoprogenitors to terminally differentiated osteoblasts). We also investigated the effect of daidzein on bone morphogenetic protein (BMP) production in osteoblasts that display the mature osteoblast phenotype. The results indicated that BMP2 synthesis was elevated significantly in response to daidzein (the mRNA increased 5.0-fold, and the protein increased 7.0-fold), suggesting that some of the effects of daidzein on the cell may be mediated by the increased production of BMPs by the osteoblasts. In conclusion, daidzein has a direct stimulatory effect on bone formation in cultured osteoblastic cells *in vitro*, which may be mediated by increased production of BMPs in osteoblasts.

Keywords: Daidzein; Estrogen; Osteoblasts; Cell viability; Cell differentiation; BMPs

1. Introduction

Osteoporosis, like menopausal symptoms, has been linked to decreased gonadal steroid production. Common sites of fracture among postmenopausal women are the vertebrae, forearm, and hip. Such fractures occur much less frequently in men of a similar age. ERT has been shown to effectively reduce the risk of osteoporosis in postmenopausal women [1]; however, because of safety concerns, ERT cannot be applied to healthy women or to women with a previous diagnosis of breast cancer, endometrial cancer, or ovarian cancer, all of which limit the clinical usage of estrogen [2,3]. A better pharmaceutical drug that is safer than estrogen is needed.

Daidzein is a natural isoflavonoid found in *Leguminosae*, and belongs to the family of diphenolic compounds with structural similarities to natural and synthetic estrogens and anti-estrogens [1]. Daidzein has been shown to bind the ER, albeit weakly compared with estradiol. Both estrogenic and anti-estrogenic effects of daidzein have been observed [1]. The estrogenic effects of the phytoestrogens have been proposed to prevent bone resorption and to increase bone density. Osteoblasts are the most important cells in bone tissues and are critical for bone formation and normal bone density. The cellular events involved in bone formation include the chemotaxis, proliferation, and differentiation of osteoblast precursors [4]. During differentiation *in vitro*, osteoblast phenotypic markers appear in the following order: accumulation of collagenous matrix, expression of ALP, secretion of osteocalcin, and finally mineralization of bone nodules [5–7]. Cultured osteoblasts have been used widely as a useful tool for analyzing osteoblast proliferation and differentiation [7]. More recently, daidzein was shown

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Abbreviations: ALP, alkaline phosphatase; BMP, bone morphogenetic protein; ER, estrogen receptor; ERT, estrogen replacement therapy; GAPDH, glyceraldehyde-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT–PCR, reverse transcription–polymerase chain reaction; and TGF-β, transforming growth factor-β.

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to stimulate protein synthesis, ALP activity, and DNA content in the osteoblast MC3T3-E1 cell line [8,9]. It has also been demonstrated that daidzein causes a significant increase of ALP activity, DNA, and calcium content in bone tissues [10]. Although the available data have demonstrated that daidzein stimulates the early differentiation of osteoblast cells, the effects of daidzein on the terminal differentiation and mineralization of osteoblasts, the synthesis of collagenous and noncollagenous proteins (including the osteoblast-specific noncollagenous protein osteocalcin), or osteoblast viability have not been investigated fully.

BMPs, which belong to the TGF-β superfamily, form dimers that are interconnected by seven disulfide bonds. Fifteen BMPs have currently been identified, and are further divided into subfamilies according to their amino acid sequences [11]. Members of each subgroup have been shown to possess osteoinductivity. Studies *in vivo* and *in vitro* have demonstrated the applicability of insulin-like growth factors (IGFs), TGF-β, and BMPs to systemically increase bone formation, promote fracture healing, and induce bone growth around implants [12]. *In vitro*, BMPs induce osteoblast differentiation of various types of cells including undifferentiated mesenchymal cells, bone marrow stromal cells, and preosteoblasts. BMPs not only inhibit myogenic differentiation, but also convert the differentiation pathway of some myogenic cells into an osteoblast lineage [13,14].

A number of systemic factors have been shown to affect BMP activity. Glucocorticoids increased osteoinductivity of BMP2, BMP4, and BMP6 10-fold in secondary rat calvarial cell cultures [15]. A stimulatory effect of glucocorticoids on BMP2 effectiveness was also shown in MC3T3-E1 cells and in rat osteoblasts [16,17]. Vitamin D acted synergistically with BMP3 in human bone marrow cultures, and also enhanced the osteoinductive actions of BMP2 implanted in intramuscular sites in mice [11]. Rickard *et al*. [18] found that estrogen stimulates the production of BMP6 at both the mRNA level and the protein level, so they speculated that some of the recognized effects of estrogen on the skeleton could be mediated by an increased production of BMP6. Daidzein is an estrogenic compound in that it can bind to the ER [1]. Whether daidzein can increase the production of BMPs in osteoblasts and whether BMPs may act in a paracrine/autocrine fashion to modulate the differentiation of osteoblasts remain to be elucidated.

To further understand the influence of osteoblast differentiation by daidzein and to understand the mechanism by which daidzein exerts a stimulatory effect on osteoblast differentiation, we isolated osteoblasts from newborn Wistar rat calvarias. These cells allow the analysis of the various differentiation stages, from osteoprogenitors to terminally differentiated osteoblasts [7]. The results illustrated the effects of daidzein on osteoblast viability, ALP activities, osteocalcin synthesis, and BMP2 mRNA expression and BMP2 protein synthesis.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from the HyClone Co., trypsin was obtained from the Difco Co., and collagenase type I and the total RNA isolation system were purchased from the Gibco Co. Daidzein was purchased from the Sigma Chemical Co., *p*-nitrophenol inorganic phosphate (PNPP) from the Amresco Co., and bicinchoninic acid (BCA) protein assay reagent from the Pierce Chemical Co. The osteocalcin assay kit was purchased from the China Atomic Energy Research Institute, and the RT–PCR kit from the Takara Shuzo Co. Ltd. rhBMP2 was purchased from the China Academy of Military Medical Science, BMP2 mAb (rabbit polyclonal IgG) from Santa Cruz Biotechnology, and peroxidase-conjugated goat anti-rabbit IgG from the DAKO Co. Plastic tissue culture dishes were purchased from Nunc Denmark. Other materials were commercial products of the highest grade available.

2.2. Osteoblast cell culture

Osteoblasts were isolated from calvarias of newborn Wistar rats by sequential enzymatic digestion as described previously [7]. The cells were cultured in DMEM containing 10% FBS and antibiotics (100 IU/mL of penicillin G and 100 μg/mL of streptomycin) and incubated at 37° in a 5% CO_2 humidified atmosphere. The medium was changed every 3 days. Daidzein was initially dissolved in 50% DMSO and then diluted with serum-free medium, so that the final DMSO concentration was 0.5% in the conditioned medium [7,19,20]. The conditioned medium was serum-free and supplemented with various concentrations of the test agents.

2.3. MTT assay

The MTT assay [reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product] was used to estimate cell viability. Cells were incubated with 0.5 mg/mL of MTT in the last 4 hr of the culture period tested. The medium was then decanted, formazan salts were dissolved with 200 μL of DMSO, and the absorbance was determined at 490 nm using an ELISA reader [21–31].

2.4. Measurement of protein concentration and ALP activity

Osteoblasts were maintained in culture flasks for 10 days, and then were seeded into 96-well Nunc plates at a density of 5×10^3/well and cultured for 24 hr. The agent to be tested was added to the wells and incubation was

continued for 2 days. The cells were then washed with physiological saline three times, and the cellular protein concentration was determined by incubation in BCA protein assay reagent containing 0.1% Triton X-100 for 30 min at 37°. The reaction was stopped by adding 1 M NaOH, and the absorbance was measured at 550 nm.

The ALP activity in the cells was assayed after appropriate treatment periods by washing the cells with physiological saline three times. ALP activity in the cells was then measured by incubation in 0.1 M $NaHCO_3$–Na_2CO_3 buffer, pH 10, containing 0.1% Triton X-100, 2 mM $MgSO_4$, and 6 mM PNPP for 30 min at 37°. The reaction was stopped by adding 1 M NaOH, and the absorbance was measured at 405 nm [32]. The percent changes of the ALP activity with respect to the value found in the control were calculated according to the formula: M = value of absorbance at 405 nm/value of absorbance at 550 nm. Percent change = $[(\text{M of the test} - \text{M of the control})/\text{M of the control}] \times 100$ [31].

2.5. Measurement of osteocalcin synthesis

The osteocalcin concentrations secreted into the culture medium were determined by radioimmunoassay using an osteocalcin assay kit. Osteoblasts were maintained in culture flasks for 15 days. The culture medium was changed 24 hr before collection, and the amounts of osteocalcin accumulated during this period were measured [5,7].

2.6. RT–PCR analysis

Expression levels of BMP2 mRNA were examined using RT–PCR. We also performed RT–PCR for GAPDH independently as an internal control. RNA was isolated with TRIzol reagent from cells cultured in 25-cm^2 Nunc plates for 5 days. The total harvested RNA was measured using a UV/Vis spectrophotometer at 260 nm. Aliquots (2 μg) of total RNA were reverse transcribed to cDNA using AMV reverse transcriptase. The generated cDNA was used as a template for PCR, which was performed with 2 μL of the template, 0.5 μL of *Taq* DNA polymerase, and 20 pM of each primer in a 50 μL reaction mixture for 30 cycles in a minicycler. Each cycle was held at 94° for 60 sec, 53° for 30 sec, and 72° for 60 sec. The oligonucleotide primers used are listed in Table 1.

After amplification, each sample was applied to a 1% agarose/ethidium bromide gel for electrophoresis. The resulting gel was photographed under ultraviolet illumination. Each RT–PCR product identified was compared to those obtained by amplifying GAPDH cDNA from the same sample. Band intensities were evaluated by densitometry.

2.7. Western blot analysis

To estimate the amount of secreted BMP2, western blot analysis was performed using BMP2 mAb (rabbit polyclonal IgG). After 5 days of treatment in serum-free medium with daidzein, osteoblast cell-conditioned medium was concentrated by ~20-fold using a freeze dryer (Christ). Aliquots of cell-conditioned medium were suspended in electrophoresis buffer and electrophoresed in 15% SDS–PAGE under reducing conditions. The blots were then electrotransferred for 90 min onto a nitrocellulose membrane (Pall Gelman Laboratory). All subsequent steps were performed at room temperature. The blots were blocked for 2 hr in Tris–HCl buffer (pH 7.5) containing 2% (w/v) BSA (blocking buffer), washed with Tris–HCl buffer (pH 7.5) three times, and probed with BMP2 mAb [rabbit polyclonal IgG, 1 μg/mL in Tris–HCl buffer (pH 7.5) containing 0.5% (w/v) BSA] for 90 min. The blots were then washed twice with Tris–HCl buffer (pH 7.5) and incubated in Tris–HCl buffer (pH 7.5) containing 0.5% (w/v) BSA for 90 min with a peroxidase-conjugated goat anti-rabbit IgG. After three washes in Tris–HCl buffer (pH 7.5), immunoreactive proteins were visualized according to "Molecular Cloning: A Laboratory Manual" (2nd Ed., Cold Spring Harbor Laboratory Press, 1989). Band intensities were evaluated by densitometry.

2.8. Statistical analysis

Data are expressed as means ± SEM. Statistical differences were analyzed using Student's *t*-test; *P* values of less than 0.05 were considered to be significant.

3. Results

3.1. Effect of daidzein and rhBMP2 on osteoblast viability

The influence of daidzein and rhBMP2 on osteoblast viability was assessed, using the MTT assay (Figs. 1 and 2). Cell viability in the presence of daidzein was significantly

Table 1
PCR primer sequences

Target	Primer sequences	PCR product size (bp)	Reference
GAPDH	5′-GGCAAGTTCAATGGCACAGT-3′ 5′-AAGGTGGAGGAATGGGAGTT-3′	725	[33]
BMP2	5′-GA(T/C)TT(T/C)(T/A)(C/G)IGA(T/C)GTIGGITGGAA-3′ 5′-CAICC(T/C)TCIACIACCAT(T/C)TC(T/C)TG-3′	272	[34]

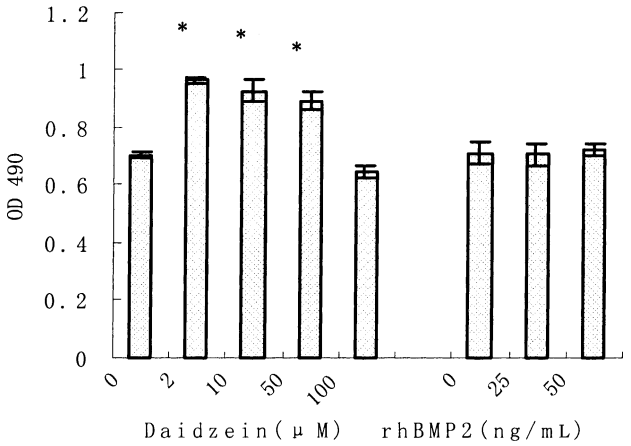

Fig. 1. Effect of daidzein and rhBMP2 on osteoblast viability. The cells were incubated with daidzein or rhBMP2 for 6 days. Each value is the mean ± SEM of 6 dishes. Key: (*) $P < 0.05$, compared with the control value without daidzein.

higher on day 6 relative to the control cultures grown in the absence of daidzein. The maximum stimulatory effect on cell viability was achieved at a concentration of 2 μM (Fig. 1), but the increase was not seen on days 2 or 4; it could only be seen after 6 days of culture (Fig. 2). There was no stimulation with the rhBMP2.

3.2. Effect of daidzein and rhBMP2 on ALP activity in osteoblasts

ALP activity is a phenotypic marker for the mature osteoblast. The effect of daidzein and rhBMP2 on the differentiation of osteoblasts was studied by determining ALP activity in the osteoblasts (Fig. 3). Cultures in the presence of 10 and 100 μM daidzein for 48 hr experienced a significant increase in ALP activity. rhBMP2 at 25 and 50 ng/mL induced increases of 1.4- and 1.5-fold, respectively.

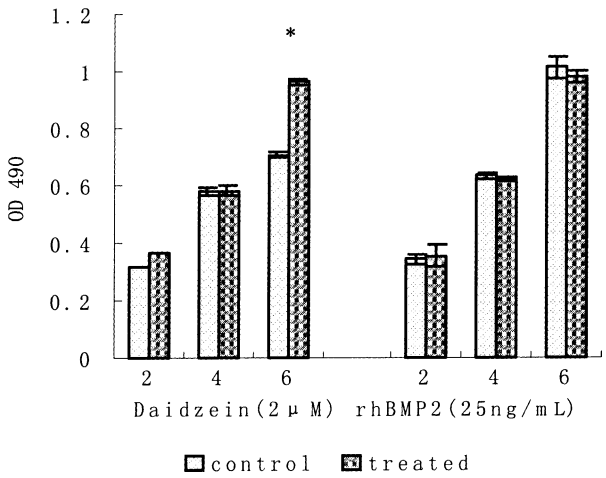

Fig. 2. Time-dependent effects of daidzein and rhBMP2 on osteoblast viability. The cells were incubated with daidzein or rhBMP2 for 2, 4, or 6 days. Each value is the mean ± SEM of 6 dishes. Key: (*) $P < 0.05$, compared with the control value without daidzein.

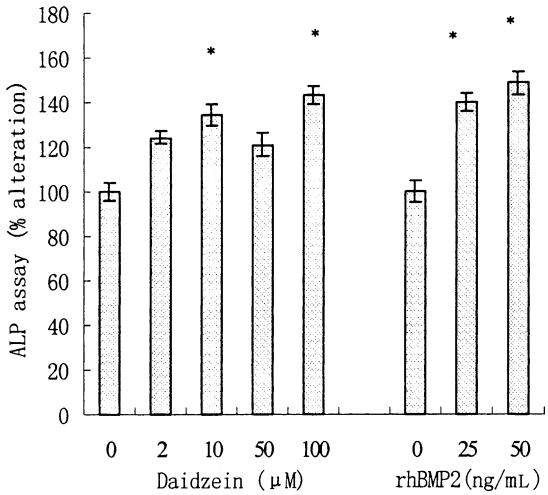

Fig. 3. Effect of daidzein and rhBMP2 on ALP activity in osteoblastic cells. The cells were incubated with daidzein or rhBMP2 for 2 days. Data points are the means ± SEM of 6 dishes. Key: (*) $P < 0.05$, compared with the control value without daidzein or rhBMP2.

3.3. Effect of daidzein and rhBMP2 on osteocalcin synthesis in osteoblasts

The effects of daidzein and rhBMP2 on the terminal differentiation of osteoblasts were also studied by determining the osteocalcin concentrations in the culture medium (Fig. 4). Osteocalcin is a specific cell marker protein for the terminal differentiation of osteoblasts, which is secreted into the culture medium by the osteoblasts. Osteoblasts treated with daidzein or rhBMP2 for 48 hr experienced a significant increase in osteocalcin synthesis. Furthermore, daidzein stimulated osteocalcin synthesis in a concentration-responsive manner, with the maximum stimulation at 100 μM.

3.4. Effect of daidzein on the expression of BMP2 mRNA in osteoblasts

As shown above, the production of osteocalcin was stimulated by daidzein. BMP2 has also been shown to play

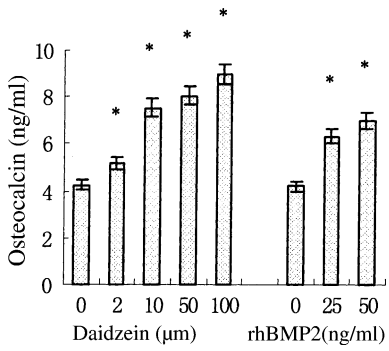

Fig. 4. Effect of daidzein and rhBMP2 on osteocalcin synthesis in osteoblastic cells. The cells were incubated with daidzein or rhBMP2 for 2 days. Each value is the mean ± SEM of 6 dishes. Key: (*) $P < 0.05$, compared with the control value without daidzein or rhBMP2.

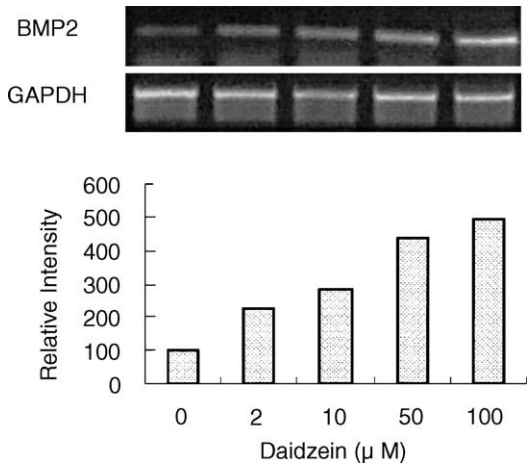

Fig. 5. Effect of daidzein on BMP2 mRNA synthesis in osteoblastic cells. The cells were incubated with daidzein for 5 days. Osteoblasts treated with daidzein for 5 days experienced a significant increase in BMP2 mRNA synthesis. The levels of BMP2 mRNA increased with increased daidzein concentration. Band relative intensities were evaluated by densitometry. Data from three independent experiments were similar.

an important role in bone formation, so the expression of the *BMP2* gene was examined using RT–PCR in the cultures grown in both the presence and the absence of daidzein for 5 days (Fig. 5). The GAPDH mRNA level was analyzed in the same samples as a reference gene. Compared to the GAPDH mRNA level, the relative levels of the BMP2 mRNA were higher in the cultures grown in the presence of daidzein than in the control cultures, as can be seen on the ethidium bromide-stained agarose gels. The maximum stimulatory effect on BMP2 mRNA expression was achieved at 100 μM.

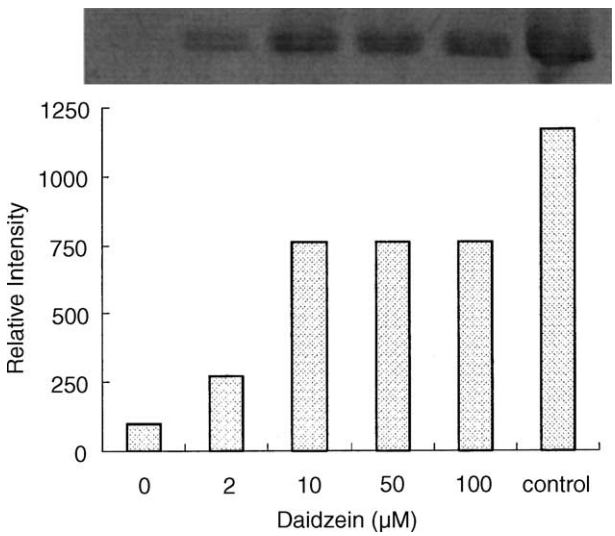

Fig. 6. Effect of daidzein on BMP2 protein synthesis in osteoblastic cells. BMP2 protein concentration in conditioned medium of osteoblasts, as measured by western blot analysis. Cells were cultured for 5 days in conditioned medium containing control and daidzein. The last lane contained recombinant human BMP2 (rhBMP2) as a positive control. The upper panel shows the appearance of the immunoreactive bands, the density of the bands relative to control as assessed by scanning densitometry. Results were similar in each of two independent experiments.

3.5. Western blot assay

Secretion of BMP2 protein was assessed by western blot analysis of conditioned medium from cultured primary osteoblasts. Under reducing conditions, the polyclonal anti-BMP2 antibody detected an immunoreactive band. Recombinant human BMP2 was loaded as a positive control. As assessed by densitometry, daidzein at 2 μM induced a 2.5-fold increase, and at 10, 50, and 100 μM induced a 7.5-fold increase (Fig. 6).

4. Discussion

Recently, isoflavonoids have received much more attention since more and more studies in humans, animals, and cell culture systems have suggested that isoflavonoids play an important role in the prevention of menopausal symptoms, osteoporosis, cancer, and heart disease [1]. Our previous studies demonstrated that the isoflavonoids may have a significant effect on the inhibition of tumor cell growth [35]. Genistein and daidzein are both natural isoflavonoids found in *Leguminosae*. Studies on the effect of genistein on bone metabolism have been reported [36–38]. More recently, daidzein was shown to stimulate bone formation and mineralization in osteoblastic MC3T3-E1 cells *in vitro* [10].

These studies demonstrated that daidzein can stimulate the early differentiation of osteoblasts, but the effects of daidzein on osteoblast viability, terminal differentiation, and mineralization have not been determined. The present study utilized primary osteoblast cultures to systematically analyze the effect of daidzein on osteoblastic cells *in vitro*. The result showed that daidzein enhanced osteoblast viability and stimulated their differentiation, from osteoprogenitors to terminally differentiated osteoblasts. The present study also analyzed the possible mechanism of the anabolic effect of daidzein on bone metabolism.

Cultures in the presence of daidzein for 6 days experienced a significant increase in cell viability that was not seen on days 2 or 4. Sugimoto and Yamaguchi [9] found that daidzein increases the DNA content in osteoblastic MC3T3-E1 cells, and speculated that it stimulates cell proliferation. The present result is consistent with that notion.

Osteoblasts cultured for 2 days in the presence of different concentrations of daidzein exhibited a significant increase in ALP activity and osteocalcin secretion. Wada *et al*. [7] reported that ALP activity in osteoblasts isolated from rat calvarias increased gradually during the culture period with a marked increase on day 20 compared to that on day 5. Compared to ALP, osteocalcin appeared much later, and was not detectable in the culture medium until day 15 [7]. Thus, we added the test agent to the medium on day 10 and measured ALP activity on day 12. Experiments were also conducted with the test agent added on day 15 with the osteocalcin concentration determined on day 17.

The results indicated that the presence of daidzein caused a significant increase in ALP activity and osteocalcin synthesis. As the appearance of ALP activity is an early phenotypic marker for mature osteoblasts, our results suggested that the presence of daidzein stimulated an early stage of osteoblast differentiation. In contrast, osteocalcin is a phenotypic marker for a later stage of osteoblast differentiation, one that coincides with mineralization, and it is one of the major noncollagenous proteins specific to mineralized connective tissues of vertebrates [39–41]. In summary, these results indicated that daidzein stimulated osteoblast differentiation at various levels from the osteoprogenitor stage to the terminal differentiation stage.

The results also demonstrated that daidzein has an anabolic effect on bone metabolism, but little is known about the mechanism of this effect. Sugimoto and Yamaguchi [9] observed that daidzein elevates cellular protein content and that ALP activity is inhibited completely by the presence of the anti-estrogen tamoxifen. They speculated that the effects of daidzein are mediated partly through the mode of action of estrogen in osteoblastic MC3T3-E1 cells, but nothing further is known about the mechanisms. The present study considered whether systemic hormones and local factors are linked to cell viability and differentiation and showed for the first time that BMPs may play an important role in the differentiation effect of daidzein on osteoblasts.

BMPs belong to the TGF-β superfamily, and are osteoinductive [11]. Although the cellular events involved in bone formation may be modulated by systemic hormones, such as alciotropic hormones, parathyroid hormone, 1,25-dihydroxyvitamin D, and sex steroids (including estrogen), the events probably are modulated predominantly by local factors or cytokines, such as BMPs, hedgehogs, the transcription factor, and core-binding factor α-1 (Cbfa1) [4,12–14]. Of the local factors, BMPs are the most potent regulators of osteoblast differentiation [12–14]. In the BMP subfamily, BMP2 was the earliest BMP detected in condensing prechondrocytic mesenchyme of developing limb buds [11,18]. BMP2 has demonstrated a strong osteoinductive capacity *in vivo* and *in vitro* [21]. The present study showed that rhBMP2 induced osteoblast differentiation based on increased ALP activity and osteocalcin expression. The present study also showed that BMP2 mRNA and protein synthesis in the cultured osteoblasts increased in response to daidzein in the culture medium.

The synthesis of BMPs can be modulated by some systemic factors [11]. Rickard *et al.* [18] reported that estrogen selectively stimulates the production of BMP6 in the estrogen-responsive human osteoblastic cell line and speculated that some of the recognized effects of estrogen on the skeleton could be mediated by increased production of BMP6, which may then act in a paracrine/autocrine fashion to modulate the differentiation of uncommitted progenitors to oseoblasts and in the immature skeleton to chondrocytes.

Daidzein is an estrogenic compound that can bind to the ER [1], which may regulate the production of one or more of the BMPs in osteoblasts. Like estrogen, BMPs are osteoinductive and directly stimulate osteoblast differentiation. In cellular physiology, daidzein acts by regulating the synthesis of local factors (including BMPs), which may then act in a paracrine/autocrine fashion to modulate the differentiation of osteoblasts. To confirm whether BMP2 expression is influenced by the presence of daidzein, we examined the expression of the *BMP2* gene using RT–PCR and of BMP2 protein synthesis in the presence and absence of daidzein using western blotting. The result indicated that daidzein caused a significant increase in BMP2 mRNA and protein synthesis in osteoblastic cells when cultured *in vitro*. The result also supports the idea that daidzein stimulates osteoblast viability and differentiation and that the effect could be mediated by local factors (including BMPs). The locally synthesized factors then act in a paracrine/autocrine fashion to modulate the viability and differentiation of osteoblasts.

In conclusion, we have demonstrated that daidzein has an anabolic effect in primary cultures of osteoblasts. Daidzein may be able to stimulate osteoblastic bone formation, and the effects may be mediated by an increased production of BMPs.

Acknowledgments

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