Genistein affects osteoblastic MC3T3-E1 cells both through estrogen receptor and BMP-Smad signaling pathways

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ABSTRACT  Many epidemiological studies show that genistein intake is effective for maintaining bone mineral density (BMD). Because the reason for the efficacy of genistein as a bone protective agent in vivo remains unclear, we investigated the mechanisms underlying the effects of genistein on BMD in relation to BMP-Smad signaling systems. When osteoblastic MC3T3-E1 cells were exposed to 1 µM genistein, they increased in number. Combined administrations of 1 µM genistein and 1 µM of ICI 182,780 inhibited the increase in cell numbers. Alkaline phosphatase (ALP) and Alizarin red staining showed high activities, indicating that genistein might promote estrogenic differentiation of MC3T3-E1 cells. Moreover, ELISA determined that production of osteoprotegerin (OPG), which is expressed by osteoblasts, was higher when 1 µM genistein was added to the medium than in controls. In contrast, when 10 ng/mL of noggin was administered in the medium, OPG production was inhibited. In order to clarify the underlying mechanism, we investigated the BMP-Smad signaling pathway. When genistein was added to the medium, it induced gene expression of BMP-4. Immunofluorescence staining showed that genistein induced phosphorylation of Smad 1/5, a downstream molecule of BMP. When noggin, which binds to BMP and blocks BMP signaling, was added to the medium, phosphorylation of Smad 1/5 was reduced. These results indicate that genistein may regulate bone metabolism through the BMP-Smad signaling pathway as well as through the estrogen receptor pathway.

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Key words : Genistein, Osteoprotegerin, BMP-Smad signaling, MC3T3-E1 cells

INTRODUCTION  Even after adolescence, bone is continuously destroyed and formed in order to maintain and repair itself and maintain body homeostasis 1), a process called bone remodeling. Osteoclasts and osteoblasts are the main cells responsible for this process. Osteoblasts exist in the surface of bone and are differentiated from mesenchymal stromal cells in response to bone morphogenetic protein (BMP), Runx2, and osterix 2). MC3T3-E1 cells, derived from neonatal mouse calvariae, are well established as an in vitro model of osteoblast differentiation 3).
When various ligands bind to osteoblast receptors, osteoblasts produce proteins such as type I collagen, osteopontin, osteocalcin (OC), and alkaline phosphatase (ALP)\(^4\). These proteins can be used as differentiation markers of osteoblasts. On the other hand, osteoclasts, which are differentiated from hematopoietic stem cells, are formed by cell surface contact with osteoblasts. The receptor activator of the nuclear factor $\kappa B$ ligand (RANK ligand; RANKL) has been identified on the osteoblast surface; it binds to RANK on the cell surface of osteoclast precursors\(^5\). Cell-to-cell interaction between osteoblasts and osteoclast precursors is necessary to activate osteoclasts. Osteoprotegerin (OPG), which is produced by osteoblasts, is an inhibitory factor in osteoclastogenesis that competes with RANK\(^6\). Together, these molecules regulate bone remodeling.

Osteoporosis is one of major causes of fracture in postmenopausal women. A decrease of estrogen level has been reported to accelerate bone loss\(^7\). In order to minimize the effects of postmenopausal bone loss, it is necessary to achieve as high a bone mineral density (BMD) as possible in adolescence\(^8\). Many epidemiological studies show that supplemental intake of various vitamins and phytoestrogens help maintain BMD\(^9\). Soy is a component of several traditional foods in Japan. Regular intake of MK-7, a vitamin K\(_2\) analogue, in fermented soybeans (natto) contributes to the maintenance of bone health\(^10\). Soybean isoflavones, which have a structural similarity to estrogen, also promote the maintenance of BMD\(^11\). Isoflavones consist mainly of genistein and daidzein, and the biological activity of genistein is higher than that of daidzein\(^12,13\).

When BMP-4 binds to receptors on the osteoblast surface, the downstream molecules Smad 1, 5, and 8 are phosphorylated\(^14\). Phosphorylated Smads 1/5/8 form heteromeric complexes with Smad 4, and these complexes are subsequently transported into the nucleus. Thus, the BMP-4 signal expresses an osteoblast-specific physiological function\(^15\). Noggin binds to BMP and is recognized as an inhibitor of BMP signaling\(^16\). The noggin/BMP interaction prevents BMPs from binding to their cell surface receptors, and disables the initiation of BMP signal transduction in target cells.

In the present study, the effects of genistein on bone metabolism were investigated in relation to BMP-Smad signaling systems.

**MATERIALS AND METHODS**

**Reagents**

Genistein and 17 $\beta$-estradiol were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) and ICI 182,780, an estrogen receptor antagonist, was obtained from Tocris Cookson Ltd. (Bristol, UK). They were dissolved in dimethyl sulfoxide (DMSO) to give a stock solution at 100 $\mu$M. Fetal bovine serum (FBS) and Eagle’s Minimal Essential Medium $\alpha$-modification ($\alpha$-MEM) were obtained from Gibco Life Technologies Co., (Carlsbad, CA, USA). $\alpha$-MEM is free of phenol red which has an estrogen-like effect. Bone morphogenetic protein (BMP) 4 and noggin were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Anti-phospho-Smad 1/5/8 antibody was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Osteoprotegerin (OPG) was measured using an enzyme-linked immunosorbent assay (ELISA) kit (RayBiotech, Inc., Norcross, GA, USA). Other materials were commercial products of the highest grade available.

**Cell culture**

Osteoblastic MC3T3-E1 cells derived from the calvaria of a newborn C57BL/6 mouse were obtained from the Riken Cell Bank and used in the 3\(^{rd}\) to 5\(^{th}\) passages. The cells were maintained in $\alpha$-MEM containing 10% (v/v) FBS. All cells were plated at a density of 2.0 x 10\(^5\) cells in 100-mm culture dishes at 37° C in a humidified atmosphere containing 5%
When cells reached subconfluence, various concentrations of genistein (0.1 µM – 100 µM) and/or ICI 182,780 (0.01 µM – 10 µM) were added to the medium. After 24 h and 96 h, the cells were counted.

**Estrogenic effect of genistein**

Subconfluent MC3T3-E1 cells were transiently transfected with reporter plasmids containing estrogen response element – chloramphenicol acetyl transferase (ERE–CAT). Transfected cells were co-cultured with the medium, 0.1 µM 17β-estradiol, 1 µM genistein, and/or 1 µM ICI 182,780. To determine the number of copies of the targeted DNA in the samples, a relative standard curve (concentration-threshold cycle) was generated by the dilution of cDNA from the calibrator. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels in the samples. The primers used in the present study were BMP-4 (forward: 5’-AGGAGGGAGGAGGAGCAG-3’, reverse: 5’-CACCTCATTCCTCGGGATGC-3’); osteocalcin (OC) (forward: 5’-TCTCTGACTCTGCTGG-3’, reverse: 5’-GTGGTGCCATAGATGCGCT-3’); ALP (forward: 5’-CGGGACTGCACTCGGATAA-3’, reverse: 5’-ATTCCACGCACGGTTCTGTTC-3’); OPG (forward: 5’-TGCAGAGAGTGTAGAGAGG-3’, reverse: 5’-CAAGGTGTCTTGGTCTCCA-3’); GAPDH (forward: 5’-TGCAGAGAGTGTAGAGAGG-3’, reverse: 5’-CAAGGTGTCTTGGTCTCCA-3’); and OPG (forward: 5’-TGCAGAGAGTGTAGAGAGG-3’, reverse: 5’-CAAGGTGTCTTGGTCTCCA-3’); GAPDH (forward: 5’-TGCAGAGAGTGTAGAGAGG-3’, reverse: 5’-CAAGGTGTCTTGGTCTCCA-3’); and OPG (forward: 5’-TGCAGAGAGTGTAGAGAGG-3’, reverse: 5’-CAAGGTGTCTTGGTCTCCA-3’). Gene expressions were examined at 2 and 24 h after genistein administration.

**Alizarin red S staining for measurement of mineralization**

To assay mineralization, osteoblasts were cultured for 28 d. Genistein (1 µM) was administered every three days. On the third, 14th, and 28th day of culture, the cells were rinsed three times with phosphate buffered saline (PBS), with 90% ethanol, and then fixed in 70% ice-cold ethanol for 20 min at 4° C. The cells were stained with 1% Alizarin red S (Sigma-Aldrich) for 5 min at room temperature and washed five times with deionized water. The stained cells were then dehydrated with 70% ethanol for 15 min and counted manually using a microscope.

**Quantification of various molecules using real-time PCR**

Total RNAs were prepared using an RNeasy Plus Mini kit (Qiagen, Tokyo, Japan). First strand cDNA was synthesized from 5 µg of total RNA using a commercial Advantage RT-for-PCR kit (Clontech Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer’s instructions. Real-time PCR was performed using the MX3000P (Stratagene, La Jolla, CA, USA). Thermocycling was performed in a final volume of 20 µL containing 10 µL of Brilliant II Fast SYBR Green PCR Master Mix (Stratagene), 1 µL of cDNA, and 5 pmol/L of each primer. To determine the number of copies of the targeted DNA in the samples, a relative standard curve (concentration-threshold cycle) was generated by the dilution of cDNA from the calibrator. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels in the samples. The primers used in the present study were BMP-4 (forward: 5’-AGGAGGGAGGAGGAGCAG-3’, reverse: 5’-CACCTCATTCCTCGGGATGC-3’); osteocalcin (OC) (forward: 5’-TCTCTGACTCTGCTGG-3’, reverse: 5’-GTGGTGCCATAGATGCGCT-3’); ALP (forward: 5’-CGGGACTGCACTCGGATAA-3’, reverse: 5’-ATTCCACGCACGGTTCTGTTC-3’); OPG (forward: 5’-TGCAGAGAGTGTAGAGAGG-3’, reverse: 5’-CAAGGTGTCTTGGTCTCCA-3’); GAPDH (forward: 5’-TGCAGAGAGTGTAGAGAGG-3’, reverse: 5’-CAAGGTGTCTTGGTCTCCA-3’); and OPG (forward: 5’-TGCAGAGAGTGTAGAGAGG-3’, reverse: 5’-CAAGGTGTCTTGGTCTCCA-3’). Gene expressions were examined at 2 and 24 h after genistein administration.

**Osteoprotegerin measurement by ELISA**

MC3T3-E1 cells were seeded onto 100-mm diameter microplates at a density of 2.5 x 10^5/well and stimulated with 1 µM genistein with or without 10 ng/mL noggin. After 96 h, the production of osteoprotegerin (OPG) in the culture supernatant was measured using a commercially available mouse OPG ELISA kit (RayBiotech, Inc.).
Immunofluorescence staining for phospho-Smad 1/5 nuclear translocation

MC3T3-E1 cells were seeded onto glass coverslips at a density of $1.0 \times 10^5$ cells/well and incubated with genistein in the presence or absence of noggin for 48 h. After 2 h incubation with 1 µM genistein or 0.5 ng/mL BMP-4, the cells were fixed in chilled 4% paraform aldehyde (PFA) for 10 min and washed with PBS. Then, cells were incubated in blocking solution containing 2% goat serum, 3% BSA and 0.2% Tween 20 in PBS for 30 min and with rabbit monoclonal anti-phospho-Smad 1/5 antibody (#9516S, Cell Signaling, Tokyo, Japan) at 1:100 dilution in blocking buffer at 4°C overnight. Cells were incubated with a 1:500 dilution of secondary Alexa Fluor 594 goat anti-rabbit IgG antibody (A-11037, Molecular Probes, Invitrogen) containing 1 µg/mL 4'6'-diamino-2-phenylindole solution (DAPI, Dojindo, Kumamoto, Japan). Finally, the coverslips were mounted using an antifade mounting medium containing DAPI (405 nm) and photographed using a confocal microscope equipped with a LD laser (473 nm:15 mW, 559 nm:15 mW) source of phase-contrast microscopy (Olympus, Tokyo, Japan). Images were taken using an All-in-One Fluorescence Microscope BZ-8000 (Keyence Japan, Osaka, Japan).

Statistical analysis

All values were expressed as means ± standard deviation. In order to compare the differences between control and administration groups, one-way ANOVA was employed using the JMP9 program (SAS Corp., Cary, NC, USA).

RESULTS

Confirmation of estrogenic effect of genistein

In order to identify the estrogenic effect of genistein, activation of estrogen response element (ERE)-containing genes was measured by means of a chloramphenicol acetyl transferase (CAT) reporter construct with an upstream ERE site in MC3T3-E1 cells. The CAT assay suggested that 1 µM genistein activated transcription through binding with estrogen receptors, although its activity was approximately 38.8% of 17β-estradiol (Fig. 1). Moreover, 1 µM ICI 182,780 had slightly lower CAT activity than 1 µM genistein, although the difference was not statistically significant. This result indicated that genistein bound to estrogen receptors and might have an estrogenic effect.

Effect of genistein on cell proliferation

Changes in cell number were investigated 24 and 96 h after administration of concentrations of 0.1 or 1 µM genistein with or without ICI 182,780 (Fig. 2A, B). MC3T3-E1 cells ($2.0 \times 10^5$) were seeded in 100-mm culture dishes. No statistical difference was observed between control and genistein-administered cells 24 h after genistein administration. On the other hand, the MC3T3-E1 cells cultured with 0.1 or 1 µM genistein with 0.1 µM ICI 182,780 showed a significant decrease in cell number at 24 h compared with those without ICI 182,780. Significant increases of cell numbers were observed at 96 h after 0.1 or 1 µM genistein administration. Moreover, combined administrations of genistein and ICI 182,780 significantly decreased cell numbers compared with

![Fig. 1. Estrogenic effect of genistein. A CAT assay suggested that 1 µM genistein activated transcription through binding with estrogen receptors, although its activity was about 40% of 17β-estradiol. *: p<0.05](image-url)
genistein administration alone.

**Osteogenic effect of genistein**

In order to investigate the osteogenic effect of genistein, alkaline phosphatase (ALP) activity and alizarin red staining were examined. Twelve hours after 0.3 ng/mL BMP-4s was administered, approximately 0.55 mmol/L of ALP activity had been induced. On the other hand, 3 ng/mL BMP-4s did not induce ALP activity, while 1 ng/mL BMP-4s induced approximately 0.25 mmol/L ALP activity (Fig. 3A). These results indicated that a
low concentration of BMP-4 might induce cell differentiation, and 1 µM genistein induced ALP activity as high as 1 ng/mL BMP-4.

Because we did not add a differentiation factor to the medium, a long incubation period (28 d) was needed to observe the bone mineralization process using Alizarin red staining. Treatment with 1 µM genistein significantly enlarged the area of calcification in MC3T3-E1 cells compared with the control (Fig. 3B). Administration of 1 µM ICI 182,780 with genistein showed a tendency to decrease the area of calcification (p<0.10). In contrast, administration of 1 µM 17β-estradiol did not produce a significant difference from the control. These findings implied the genistein might induce the calcification of osteoblasts through an estrogen receptor-dependent pathway.

Induction of osteoblast-specific genes by genistein

Because genistein may induce both cell proliferation and cell differentiation, expressions of osteoblast-specific genes were examined. Expression of each gene was adjusted to the GAPDH control. When mRNA expression of the control was taken as 1.0, the relative mRNA expressions of BMP-4, ALP, OPG, and OC were 1.34, 1.08, 5.40, and 1.56, respectively, when MC3T3-E1 cells were cultured with 1 µM genistein for two hours (Fig. 4, left panel). In addition, the relative mRNA expressions at 24 h were 0.76, 1.09, 0.58, and 0.76, respectively. If changes of relative mRNA expressions between 0.5 to 2.0 times were not significant, genistein only enhanced OPG mRNA expression in early cultures.

Induction of osteoprotegerin by genistein

It is assumed that genistein induced proliferation and differentiation of osteoblasts both through estrogen-receptor and BMP-Smad signaling pathways. Because genistein induced only OPG mRNA expression among several genes related to osteogenesis, the production of OPG was measured using ELISA in MC3T3-E1 cells with or without noggin (a BMP inhibitor). As shown in Fig. 5, MC3T3-E1 cells secreted 2513.0 ± 101.0 pg/mL OPG, and this was significantly higher than that of control (without genistein, 1607.7 ± 63.6 pg/mL). Moreover, the addition of 10 ng/mL of noggin with

![Fig. 4. Relative expression of osteoblast-specific molecules after administration of 1 µM genistein. mRNA expression was adjusted to GAPDH as a control. mRNA expression of control is indicated as 1.0.](image)
genistein to the MC3T3-E1 cell culture significantly decreased OPG production of MC3T3-E1 cells (1486.3 ± 88.1 pg/mL), compared with that of OPG production due to genistein alone, and the production capacity returned to the control level. Thus, the OPG production of MC3T3-E1 cells induced by genistein might be transduced through the activation of BMP-Smad signaling pathways.

Genistein activates BMP-Smad signaling in MC3T3-E1 cells

Since genistein tended to induce BMP-4 mRNA expression and combined administration of genistein and noggin inhibited the production of OPG, the BMP-Smad signaling pathway was investigated. Nuclear localization of phosphorylated Smad (pSmad) 1/5 was measured using immunofluorescence staining in MC3T3-E1 cells cultured with BMP-4 and genistein with or without noggin. The number of pSmad 1/5-positive cells was higher in the BMP-4 or genistein-treated cells than in the control. On the other hand, pretreatment with noggin significantly reduced the number of pSmad 1/5-positive cells in the BMP-4, and genistein-treated cells (Fig. 6E, F). The pSmad-positive index of genistein-administered cells was 4.9 times higher than that of the control (Fig. 6G), and the pSmad-positive index of BMP-4-administered cells was 3.6 times higher than that of the control. Furthermore, the index induced by genistein was higher than that induced by BMP-4. In addition, when noggin was administered with genistein, the pSmad-positive index was reduced compared with when genistein was administered alone. These results indicate that genistein may affect the BMP-Smad signaling pathway directly. The administration of nogin to the MC3T3-E1 cells cultured with BMP-4 did not affect the pSmad-positive index, although noggin decreased the index in genistein-supplemented cultures. The reason why
noggin did not inhibit BMP-4 induced Smad 1/5 phosphorylation in this culture system was not clear; the exogenous BMP may have been too strong to be suppressed by noggin administration because this activation was intrinsic enough not to be suppressed by noggin.

**DISCUSSION**

Isoflavones occur naturally as glycosidic forms and need to be hydrolyzed to form isoflavone aglycones\(^{18}\). Many epidemiological studies show that dietary supplementation with isoflavone aglycones is effective to prevent bone mineral loss\(^{19}\). This effect occurs because isoflavones have a structural similarity to estrogen and act as agonists of estrogen\(^{18}\). Among various isoflavone aglycones, genistein has the highest physiological activity. In the present study, we investigated the effect of genistein on induction of osteoblast differentiation and production of OPG in MC3T3-E1 cells through not only the estrogen receptor-dependent pathway but also the BMP-Smad signaling pathway. Baicalin, one of the flavonoids, induces osteoblast differentiation through the Wnt/\(\beta\)-catenin signaling pathway\(^{20}\). Park reported that melatonin promoted osteoblast differentiation through BMP/Wnt signaling\(^{21}\). These findings suggest that the Wnt signaling pathway is essential for osteoblast differentiation. On the other hand, the Wnt signaling pathway modulates differentiation,

![Fig. 6. Nuclear localization of pSmad 1/5 by immunofluorescence staining.](image)

Nuclear localization of pSmad 1/5 is indicated by red. MC3T3-E1 cells were cultured without noggin (A-C) and with 10 ng/mL noggin (D-F). The pSmad-positive indexes calculated as (the number of pSmad 1/5-positive cells/number of total cells) were A: 19.4 ± 5.4, B: 67.4 ± 14.4, C: 94.2 ± 3.7, D: 12.2 ± 2.1, E: 56.7 ± 9.2, F: 43.7 ± 11.6 (G). **: \(p<0.01\), *: \(p<0.05\)
Hinenoya H, et al.: Induction of OPG by genistein through BMP signaling in osteoblasts

proliferation, and mineralization in bone formation as a downstream of BMPs\textsuperscript{22, 23}. Because the Wnt signaling and BMP-Smad signaling pathways are closely related to each other, we investigated the effects of genistein on the BMP-Smad signaling pathway.

In the present study, we confirmed that genistein induced pSmad\textsubscript{1/5} and that noggin reduced this induction, although noggin itself did not affect phosphorylation. Even though we did not confirm that genistein affected the Wnt signaling pathway and bound to BMP receptors directly, our results indicate that genistein might affect BMP signaling. Moreover, genistein induced ALP activity, indicating that genistein might induce cell differentiation. On the other hand, when genistein was added to the medium, the number of cells was significantly increased, and ICI 182,780 partially blocked this increment. The CAT assay revealed that genistein may bind to estrogen receptors and may have an estrogenic effect. These results indicate that genistein may affect not only cell proliferation due to stimulation of the estrogen receptor-dependent pathway but also cell differentiation by the BMP-Smad signaling pathway.

Osteoclasts are derived from hematopoietic stem cells and are differentiated by cell-to-cell contact with osteoblasts\textsuperscript{24}. One possible strategy to prevent osteoporosis is reduction of osteoclast function. OPG is produced by osteoblasts and inhibits osteoclast differentiation acting as a decoy receptor of RANKL\textsuperscript{24}. The present study revealed that genistein affected OPG production, and that this effect was inhibited by noggin. These findings may indicate that genistein might affect cell differentiation by the BMP-Smad signaling pathway. Although the affinity of genistein to estrogen receptors is about 1/1,000 that of 1\textbeta\textsubscript{-}estradiol\textsuperscript{25}, production of OPG might prevent BMD loss. Moreover, treatment with glucocorticoids results in a substantial loss of bone, whereas glucocorticoids inhibit OPG mRNA expression\textsuperscript{26}. Genistein acts as a tyrosine kinase inhibitor, and administration of genistein also succeeds in preventing glucocorticoid-induced osteoporosis\textsuperscript{27}. These findings indicate that genistein might maintain BMD through production of OPG.

Bone metastasis is observed in some cancers, and the OPG/RANK/RANKL system plays an important role in this process\textsuperscript{28}. It has been reported that 35% of patients with prostate cancer develops hematogenous metastases, of which bone metastasis is the most frequent\textsuperscript{28}. OPG inhibits the formation of RANK/RANKL interaction, so that OPG might prevent metastatic bone destruction in prostate cancer. On the other hand, genistein in combination with lower doses of docetaxel might enhance the antitumor and antimetastatic activities of docetaxel, while reducing the side effects of docetaxel. Moreover, genistein itself down-regulates some genes critical to the promotion of cell cycle progression and cell proliferation, and up-regulated some genes related to the induction of apoptosis and cell cycle arrest\textsuperscript{29, 30}. Taken together, genistein might act not only as an antitumor agent but also reduce the side effects of docetaxel.

In Japan, although the intake calcium from foods is much lower than in Western countries, the prevalence of osteoporosis is much lower\textsuperscript{31}. Moreover, it has been suggested that consumption of soy foods may contribute to the relatively low rates of breast, colon, and prostate cancers in Japan\textsuperscript{32}. Genistein possesses weak estrogenic activity, and is also a specific inhibitor of protein tyrosine kinases; it also inhibits DNA topoisomerases and other critical enzymes involved in signal transduction. These effects of genistein might promote anticancer effects. Soy consumption in Japan is much higher than in Western countries\textsuperscript{32}, and may contribute to the lower rates of osteoporosis and cancers.

This study has some limitations. First, we did not investigate the Wnt signaling pathway,
which may affect bone mineral metabolism. The relationship between the Wnt-signaling and BMP-Smad signaling pathways should be investigated. Second, although genistein affected not only cell proliferation but also cell differentiation, the timing of this switch was not investigated. Further studies should be performed in order to clarify this switching system.

In conclusion, genistein may induce osteoblast proliferation and differentiation through the BMP-Smad signaling pathway as well as the estrogen receptor-dependent pathway. These results suggest that genistein is effective to maintain bone mineral density.

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Competing interests

The authors declare that they have no competing interests.

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