— Review —

Isoflavone and Bone Metabolism: Its Cellular Mechanism and Preventive Role in Bone Loss

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Bone loss with increasing age induces osteoporosis. This loss may be due to increased bone resorption and decreased bone formation. Osteoporosis with a decrease in bone mass is widely recognized as a major public health problem. Pharmacological and nutritional factors may prevent bone loss with increasing age. The chemical compounds in food that act on bone metabolism, however, are poorly understood. Genistein is a natural isoflavonoid phytoestrogen found in *Leguminosae* and has been demonstrated to have an anabolic effect on bone metabolism, suggesting its role in the prevention of osteoporosis. Genistein has a stimulatory effect on bone formation and mineralization in the tissue culture system *in vitro*, and it can stimulate protein synthesis in osteoblastic cells. Moreover, genistein has been shown to inhibit osteoblastic bone resorption by preventing the formation and differentiation of osteoclast-like cells from bone marrow cells, and the apoptosis of mature osteoclasts is induced by genistein through the Ca^{2+} signaling mechanism. Also, the suppressive effect of genistein on rat bone osteoclasts is partly involved in the inhibition of protein kinase and the activation of protein tyrosine phosphatase in osteoclasts. Daidzein, an isoflavone, did not have a greater effect than genistein. Various polyphenols [glycitein, resveratol, quercetin, catechin, and (–)-epigallocatechin gallate] found in food and plants did not have an anabolic effect on bone calcification in tissue culture *in vitro*. Genistein may be of significance in the prevention of bone loss with increasing age. The dietary intake of isoflavone (genistein and daidzein) could prevent bone loss in ovariectomized rats which are model animals of osteoporosis. In addition, the preventive effect of genistein on bone loss with aging is enhanced by the combination of zinc or casein phosphopeptides as food factors. Genistein is a useful biofactor in the prevention of osteoporosis.

Key words —— isoflavone, genistein, daidzein, bone metabolism, osteoporosis

INTRODUCTION

Many plant foods contain small amounts of the diverse phytoestrogen molecules that have the potential to improve health. Food phytoestrogen molecules can be divided into two major chemical classes: isoflavone and coumestans. The isoflavones are found predominantly in soybeans (*Glycine max*), whereas coumestans are produced primarily by clovers (*genus Trifolium*) and some legumes. These molecules function as antioxidants in plants, but in mammaliam tissues these natural products act as agonists, or partial agonists, of estrogens. Especially,

the soybean-derived isoflavone, are receiving great scrutiny as food supplements for the purpose of both enhancing human health and preventing several common diseases, such as cardiovascular disease, cancers of reproductive tissues, and osteoporosis.[1–5] Isoflavones may be pharmacologically interesting.

Isoflavones including daidzin, daidzein, genistin, and genistein are present in soybean at a comparatively higher concentration. As shown in Fig. 1, daidzin and genistin are hydrolyzed to daidzein and genistein by β-glucosidase in the gastrointestinal system, respectively. Genistein has been shown to have a strong inhibitory effect on protein tyrosine kinases,[6,7] and it can cause cell cycle arrest and apoptosis in leukemic cells.[7,8] Such a cellular mechanism may be important in the prevention of cancers. The biological effect of genistein on cellular function, however, has not been fully clarified.

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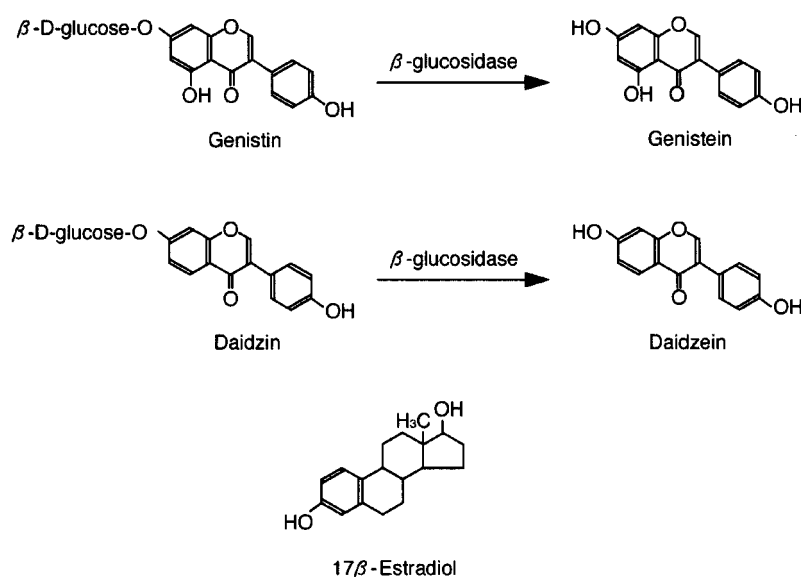
Fig. 1. Chemical Structure of Isoflavones

More recently, it has been demonstrated that genistein and daidzein have an anabolic effect on bone metabolism in rats,[9–11] suggesting their role in the prevention of osteoporosis, which is widely recognized as a major public health problem. The most dramatic expression of this disease is represented by fractures of the proximal femur, and the number of which increases as the population ages.[12,13] Malnutrition or undernutrition is often observed in the elderly, and it appears to be more severe in patients with hip fracture than in the general aging population.[14,15] Deficiency in both micronutrients and macronutrients appears to be strongly implicated in the pathogenesis and the consequences of hip fracture in osteoporotic elderly.[16] Nutritional and pharmacological factors are important in preventing bone loss with increasing age.

Isoflavones, as a food factor, are a useful tool in the prevention of and therapy for osteoporosis. This review describes mainly our findings concerning the biochemical action of genistein and daidzein on bone metabolism and the preventive effect on experimental osteoporosis.

ACTION OF ISOFLAVONE ON OSTEOBLASTIC BONE FORMATION

Anabolic Effect of Isoflavone on Bone Metabolism in Tissue Culture

Bone metabolism is regulated by functions of osteoblasts and osteoclast which are localized on bone tissues. Osteoblasts stimulate bone formation and calcification while osteoclasts promote bone resorption. The anabolic effect of genistein on bone metabolism has been investigated in tissue culture using the femoral-metaphyseal tissues obtained from elderly female rats *in vitro*.[9,10] Bone tissues were cultured for 24 hr in Dulbecco's modified Eagle's medium (high glucose, 4.5%) with bovine serum albumin (serum-free) and antibiotics containing either vehicle or genistein. The presence of genistein (10^{-6} and 10^{-5} M) was found to induce a significant increase in calcium content, alkaline phosphatase activity, which is a marker enzyme of osteoblasts, and deoxyribonucleic acid (DNA) content, which is an index of bone cell number in bone tissues, in rat femoral-metaphyseal tissues.[9] The effect of genistein in increasing bone components was equal to the stimulatory effect of 17β-estradiol. The anti-estrogen tamoxifen (10^{-7} M) was shown to completely block the genistein-induced increase in bone components, although tamoxifen itself had no effect on these components.[9] These findings suggest that the anabolic effect of genistein on bone metabolism is partly mediated through estrogen-like action. Presumably, genistein binds to the receptor of estrogen in osteoblastic cells which this receptor is localized.[17,18]

The effect of genistein and genistin on bone components is compared in culture system *in vitro*.[10] The presence of genistein (10^{-8}–10^{-5} M) or genistein (10^{-7}–10^{-5} M) was shown to increase alkaline phosphatase activity, DNA and calcium contents in the

femoral-metaphyseal tissues obtained from elderly female rats.[10] The effect of genistein (10^{-5} M) or genistin (10^{-5} M) in increasing bone components were completely blocked in the presence of cycloheximide (10^{-6} M), an inhibitor of protein synthesis in the translational process, suggesting that the anabolic effect of either isoflavone is partly based on a newly synthesized protein component.

The effect of daidzein on cortical bone of the femoral-diaphyseal tissues obtained from elderly female rats *in vitro* was investigated.[11] The presence of daidzein (10^{-6} and 10^{-5} M) in culture medium caused a significant increase in bone components. This effect was equal to that of genistein (10^{-6} and 10^{-5} M). The combination of daidzein and genistein did not have an additive effect. In addition, the stimulatory effect of daidzein on bone components was completely prevented in the presence of cycloheximide, suggesting that the daidzein's effect is dependent on osteoblastic protein synthesis like genistein action. Genistein is 5,7,4′-trihydroxyisoflavone, and daidzein is 7,4′-dihydroxyisoflavone, and the chemical structure of the two compounds is similar. Presumably, the chemical form of dihydroxyisoflavone is important in its anabolic effect on cortical bone. Genistein and daidzein may have a bone effect which operates through the same mechanism.

The effect of phosphogenistein and phosphodaidzein, which are phosphorylated for the hydroxyl group (OH) at the 7-position of genistein and daidzein, on bone components has been demonstrated.[19] At 10^{-5} M, respectively, phosphogenistein and phosphodaidzein were shown to increase bone components in tissue culture *in vitro*. The phosphoisoflavones with a lower concentration of 10^{-6} M, at which genistein and daidzein have an anabolic effect on bone components,[9,10,13] had no effect. Presumably, the OH-group at 7-position of genistein and daidzein is important in exercising an anabolic effect of isoflavone on bone components.

The effect of various polyphenols found in food and plants on bone metabolism has not yet been clarified. We determined the effect of polyphenols [glycitein, resveratol, quercetin, catechin, and (−)-epigallocatechin gallate (EGCg)] on bone calcification *in vitro*.[20] Of various polyphenols used, genistein was found to have a unique anabolic effect on bone calcification as shown in Fig. 2. Polyphenols did not have a direct effect on stimulating bone calcification *in vitro*.

Action of Isoflavone on Osteoblastic Cells

Osteoblasts play an important role in the stimulation of bone formation and calcification. The action of isoflavones on osteoblastic cells *in vitro* was shown.[21–23] Osteoblastic MC3T3-E1 cells were cultured for 48 hr in a serum-free α-minimal essential medium (α-MEM) containing genistein (10^{-7}–10^{-5} M) or daidzein (10^{-7}–10^{-5} M). The presence of genistein (10^{-6} and 10^{-5} M) or daidzein (10^{-6} and 10^{-5} M) caused a significant elevation of protein content, alkaline phosphatase activity, and DNA content in the cells.[21,23] The ability of genistein (10^{-5} M) or daidzein (10^{-5} M) to increase protein content, alkaline phosphatase activity, and DNA content in the cells was shown to be inhibited in the presence of cycloheximide (10^{-6} M), suggesting that the effect of isoflavone results from a newly synthesized protein component. Thus the anabolic effect of genistein in osteoblastic cells was not distinguishable from that of daidzein.

The effect of 17β-estradiol (10^{-9} M) in raising protein content and alkaline phosphatase activity in osteoblastic cells was not enhanced in the presence of genistein (10^{-5} M).[21] Meanwhile, cell protein content showed an additive effect of 17β-estradiol and daidzein, but their effects on alkaline phosphatase activity were not additive. Moreover, the effect of genistein or daidzein in elevating cellular protein content and alkaline phosphatase activity was clearly inhibited in the presence of tamaxifen (10^{-6} M), suggesting that the effect of the isoflavone is partly mediated through estrogen action. The receptors of estrogen are found in osteoblastic cells.[17,18] Genistein has been shown to bind to estrogen receptor β in osteoblastic cells,[24] although it has not been reported whether daidzein can bind to estrogen receptors. It is speculated, however, that daidzein may bind to estrogen receptor β in osteoblastic cells. The nuclear localization of genistein and daidzein in these cells has not been fully clarified.

DNA content in osteoblastic cells was significantly increased in the presence of genistein or daidzein, suggesting that the isoflavone stimulates cell proliferation. Also, the isoflavone can increase alkaline phosphatase activity, which is a marker enzyme in the differentiation of osteoblastic cells. Genistein and daidzein may have a stimulatory effect on the proliferation and differentiation of osteoblastic MC3T3-E1 cells.

As mentioned above, genistein and daidzein have an anabolic effect in osteoblastic MC3T3-E1 cells. The isoflavones may be able to stimulate osteoblas-
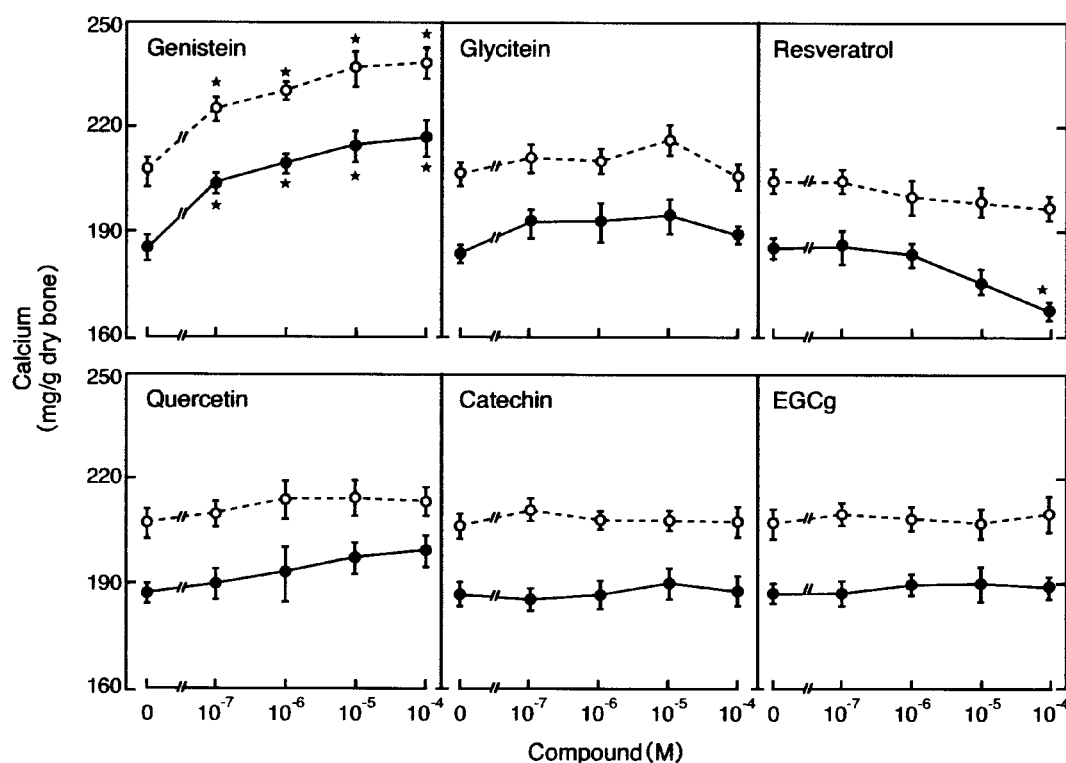
Fig. 2. Effect of Various Polyphenols on Calcium Content in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats *in Vitro*

Bone tissues were cultured for 24 hr in a serum-free medium containing either vehicle or various polyphenols (10^{-7}–10^{-4} M). Each value is the mean ± S.E.M. of five experiments with separate rats. *$p < 0.01$ compared with the control (none) value. Open circles, diaphysis; closed circles, metaphysis.

tic bone formation, supporting the observation that they have an overall stimulatory effect on bone formation and mineralization in bone tissue culture system.[9–11]

Action of Isoflavone on Protein Synthesis in Osteoblastic Cells

The cellular mechanism by which isoflavone stimulates osteoblastic bone formation has been studied in relation to protein synthesis.[23] A 5500 *g* supernatant of the homogenate of osteoblastic cells which were cultured in the presence of genistein or daidzein, was used for assay of protein synthesis with [3H]leucine incorporation *in vitro*. The presence of genistein or daidzein stimulated protein synthesis in osteoblastic MC3T3-E1 cells *in vitro*. The isoflavone's effect was blocked by the culture with actinomycin D or cycloheximide, an inhibitor of protein synthesis in the transcriptional or translational processes, suggesting that the isoflavone-induced promotion of protein synthesis in osteoblastic cells is partly based on a newly synthesized protein component.

The direct effect of isoflavones on protein synthesis is also shown.[23] The addition of genistein (10^{-7}–10^{-5} M) or daidzein (10^{-7}–10^{-5} M) into the reaction mixture of protein synthesis using the cell homogenate from osteoblastic cells cultured without isoflavone treatment caused a significant increase in protein synthesis *in vitro*. This increase was clearly blocked in the presence of cycloheximide, indicating that genistein or daidzein can directly stimulate protein synthesis *in vitro*. Moreover, either of these isoflavones was shown to increase [3H]leucyl-tRNA synthetase activity in the cytosol fraction of osteoblastic cell homogenate,[23] genistein having a greater effect than daidzein. Isoflavone could directly activate leucyl-tRNA synthetase which is a rate-limiting enzyme of the translational process of protein synthesis.[25] The cellular mechanism by which isoflavone stimulates osteoblastic bone formation is summarized in Fig. 3.

The possibility cannot be excluded that genistein or daidzein acts on the translational process in osteoblastic MC3T3-E1 cells, since the stimulatory effect of the isoflavones on protein synthesis in osteoblastic cells is clearly blocked by the treatment of actinomycin D. Genistein has been shown to bind to estrogen receptors in osteoblastic cells,[24,26] while daidzein cannot bind to estrogen receptors.[27] More-
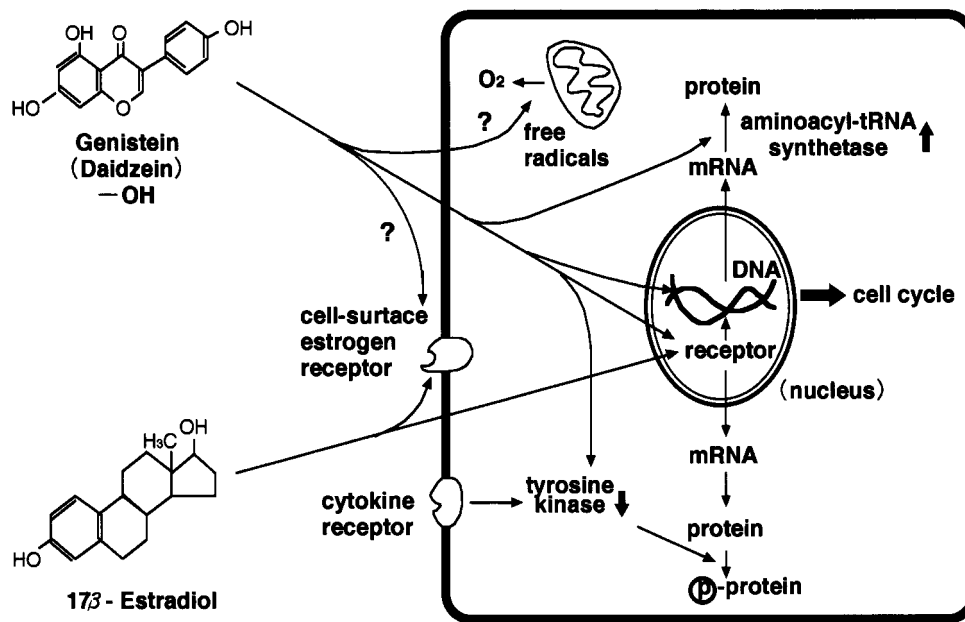
Fig. 3. Cellular Mechanism by Which Isoflavone Stimulates Osteoblastic Bone Formation

Genistein and daidzein directly activate aminoacyl-tRNA synthetase, a rate-limiting enzyme in translational process, in osteoblastic cells. The isoflavone can increase protein synthesis. It is possible that genistein may be able to enhance transcriptional activity by its binding to estrogen receptors and/or a direct effect of isoflavone on gene in the cells.

over, it may be possible that genistein or daidzein can bind to transcriptional proteins, which differ from estrogen receptors, in osteoblastic MC3T3-E1 cells. Whether or not isoflavones have an effect on various protein kinases and protein phosphatases which are related to osteoblastic cell proliferation and nuclear DNA synthesis needs to be determined.

ACTION OF ISOFLAVONE ON OSTEOCLASTIC BONE RESORPTION

Effect of Isoflavone on Bone Resorption in Tissue Culture

It has been reported that parathyroid hormone (PTH), prostaglandin E_2 (PGE$_2$), and lipopolysacharide (LPS) have a stimulatory effect on bone resorption in a culture system *in vitro*.[28–32] The presence of PTH (10^{-7} M), PGE$_2$ (10^{-5} M), and LPS (10 μg/ml) could clearly stimulate bone resorption in the femoral-metaphyseal tissues cultured for 48 hr, when bone resorption was estimated by a decrease in bone calcium content.[33] It has been shown that the concentration of bone-resorbing factors used can display a maximum effect on bone resorption in tissue culture *in vitro*.[32] The effect of bone-resorbing factors (PTH, PGE$_2$, and LPS) on stimulating bone resorption was completely inhibited in the pres-

ence of genistein (10^{-7}–10^{-5} M), indicating that the isoflavone has an inhibitory effect on bone resorption in a tissue culture system *in vitro*.[33]

PTH or PGE$_2$ has been shown to decrease in bone alkaline phosphatase activity with a corresponding increase in bone acid phosphatase activity.[34,35] The PTH- or PGE$_2$-altered alkaline and acid phosphatase activities were completely blocked in the presence of genistein.[33] Moreover, genistein produced a significant elevation of alkaline phosphatase activity in bone tissues cultured in the presence of PTH or PGE$_2$. These observations suggest that the inhibitory effect of genistein on bone resorption is partly involved in the restoration of bone phosphatase activity altered by PTH or PGE$_2$.

PTH and PGE$_2$ caused a remarkable increase in glucose consumption and lactic acid production by bone tissues.[33] The production of lactic acid from bone tissues may be related to the augmentation of glucose consumption. Presumably, PTH- and PGE$_2$-stimulated lactic acid production by bone tissues can hasten a decrease in bone calcium content, since the stimulatory mechanism of PTH on bone resorption is related to the extracellular release of acid by bone cells (osteoclasts).[36] Genistein completely blocked the PTH- or PGE$_2$-induced increase in both glucose consumption and lactic acid production by bone tissues.[33] The inhibitory effect of genistein on bone

resorption is partly related to the prevention of lactic acid production by bone tissues.

The presence of estrogen (17β-estradial) caused a complete inhibition of the PTH-decreased bone calcium content and PTH-increased bone glucose consumption.[33] The effect of estrogen was not further enhanced by genistein. Meanwhile, the inhibitory effect of genistein on the PTH-stimulated bone resorption was clearly blocked in the presence of tamoxifen, an anti-estrogen.[37] The effect of genistein to inhibit bone resorption is partly mediated through an estrogen-like action.

Genistein has a stimulatory effect on bone formation and mineralization in tissue culture *in vitro*.[9–11] Also, the isoflavone can inhibit bone resorption in tissue culture.[33] Genistein may thus have an important role in the preservation of bone mass.

Inhibitory Effect of Isoflavone on Osteoclast-Like Cell Formation

Osteoclasts, bone-resorbing cells, are formed from bone marrow cells.[38] Osteoclast-like cell formation is estimated by staining for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts.[39,40] The bone marrow cells of mouse were cultured for 7 days in α-MEM containing a well-known bone resorbing agent [PTH, PGE$_2$, 1,25-dihydroxyvitamin D$_3$ (VD$_3$), or LPS] with an effective concentration. The presence of PTH (10^{-8} M), PGE$_2$ (10^{-6} M), VD$_3$ (10^{-8} M), or LPS (1 μg/ml) induced a remarkable increase in osteoclast-like multinucleated cells.[41] These increases were significantly inhibited in the presence of genistein (10^{-7}–10^{-5} M).[41] The inhibitory effect of genistein (10^{-5} M) was equal to the effect of other anti-bone-resorbing agents (calcitonin, 17β-estradial, and zinc sulfate)[42] on osteoclast-like cell formation in mouse marrow culture. Genistein had a potent inhibitory effect at the later stage of marrow cell differentiation. The inhibitory effect of genistein on osteoclast-like MNC formation in mouse marrow culture seemed greater than that of daidzein.[41]

Genistein significantly inhibited dibutyryl cyclic adenosine monophosphate (DcAMP)-induced osteoclast-like MNC formation, whereas the isoflavone did not have an inhibitory effect on phorbol 12-myristate 13-acetate (PMA)-induced osteoclast-like cell formation.[41] PMA can directly activate protein kinase C.[43] Genistein may inhibit osteoclast-like MNC formation stimulated by the cyclic AMP signaling-dependent pathway, but not that by protein kinase C signaling. The stimulatory effect of PTH

or PGE$_2$ on osteoclast-like cell formation from mouse marrow cells has been shown to be mediated through the cyclic AMP signaling pathway.[44,45] Presumably, an inhibitory effect of genistein on osteoclast-like MNC formation induced by PTH or PGE$_2$ is partly based on the blocking action for the pathway of cyclic AMP signaling at the differentiation stage of marrow cells.

It has been reported that genistein can inhibit tyrosine kinase.[7] However, it is unknown whether genistein directly inhibits cyclic AMP-dependent kinase (A kinase) in mouse marrow cell culture. Genistein has been reported to induce the cardiac cystic fibrosis transmembrane regulator chloride current by a tyrosine kinase-independent and protein kinase A-independent pathway in guinea pig ventricular myocytes.[46] It is possible, however, that genistein may inhibit protein kinase A directly at the differentiation stage of marrow cells. In addition, if genistein can inhibit tyrosine kinase,[7] the action of the isoflavone on osteoclastic cell formation may be related, in part, to an inhibitory effect on the kinase. The cellular mechanism by which genistein inhibits osteoclast-like cell formation from marrow cells may be involved in cyclic AMP signaling, as shown in Fig. 4.

Genistein could inhibit bone resorption induced by bone-resorbing agents in tissue culture.[33] The isoflavone-induced inhibition of bone resorption may partly be based on the inhibition of osteoclastic cell formation from bone marrow cells.

Suppressive Effect of Isoflavone on Osteoclastic Cells

The effect of genistein in inhibiting osteoclast-like MNC formation from bone marrow cells of mouse[41] and rats[47] was remarkable at the later stage of cell differentiation, suggesting that isoflavone acts on pre-osteoclasts and/or osteoclasts (Fig. 4). When the bone cells isolated from rat femoral tissues were cultured for 48 hr without the addition of bone-resorbing factor, many TRACP-positive MNCs were attached to the culture dish, and the cells disappeared with calcitonin addition, a specific inhibitor of osteoclasts, indicating that the cells were osteoclasts.[48] The addition of genistein caused a significant decrease in the number of osteoclasts, suggesting that the isoflavone inhibits the attachment of cells to a culture dish and stimulates the death (apoptosis) of the cells.[47] It does not, however, exclude the possibility that the effect of genistein is partly mediated by its action on osteoblasts and/or stromal cells in
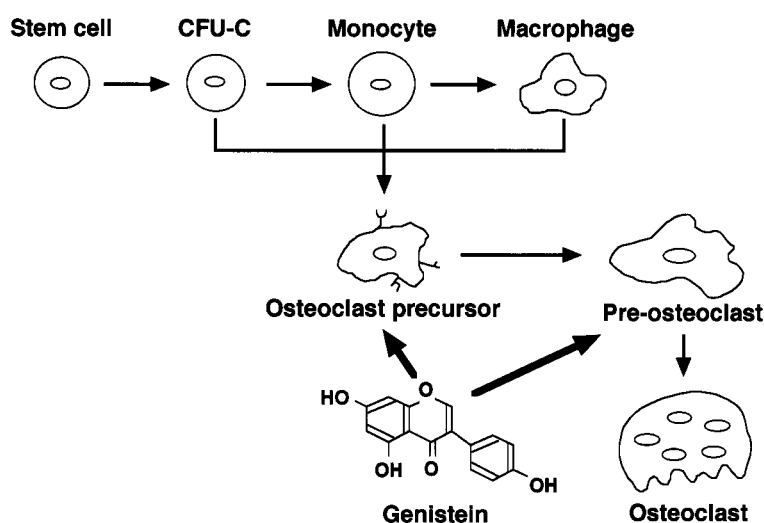
Fig. 4. Mechanism of Isoflavone Action to Inhibit Osteoclast-Like Cell Formation from Bone Marrow Cells
    Genistein uniquely inhibits the cell differential process from pre-preosteoclasts to preosteoclasts, and the isoflavone has a direct inhibitory action in osteoclasts.

bone marrow cells.

Calcitonin is known to inhibit osteoclast activity.[48] The hormonal effect may be mediated through the two pathways of cyclic AMP and Ca^{2+} signalings.[49] The addition of calcitonin, DcAMP or calcium chloride in culture medium was shown to suppress the number of osteoclasts isolated from rat femoral tissues.[47] The addition of dibucaine, an antagonist of calmodulin, or staurosporine, an inhibitor of protein kinase C, completely prevented the diminution of osteoclasts induced by calcitonin, DcAMP or calcium chloride.[47] The effect of genistein in decreasing the cells was clearly blocked by these inhibitors,[47] suggesting that the suppressive effect of isoflavone on osteoclasts is partly mediated through the pathway of Ca^{2+} signaling.

Ca^{2+} ionophore (A23187) caused a remarkable decrease in the number of osteoclasts, indicating that the entry of Ca^{2+} in the cells induces osteoclast death.[47] This effect was seen in the presence of genistein or calcium chloride. Ca^{2+} can activate endonuclease, and the metal induces apoptosis in cells.[50,51] The effect of A23187 in decreasing osteoclasts may be based on Ca^{2+}-activated DNA fragmentation, which induces apoptosis. Genistein has been reported to induce apoptosis in immature human thymocytes by inhibiting topoisomerase II.[52] Presumably, the isoflavone stimulates apoptosis of osteoclasts, and its mechanism is partly related to the pathway of Ca^{2+} signaling. It may be possible that genistein stimulates Ca^{2+} entry into osteoclasts, since it has been reported that the isoflavone can directly open a chloride channel in human cystic fibrosis transmembranes.[53] In addition, daidzein has been shown to suppress the number of osteoclasts,[47] although daidzein does not have a greater suppressive effect than genistein. The effect of daidzein was completely abolished in the presence of dibucaine or staurosporine, supporting that this effect is partly involved in Ca^{2+}-signaling mechanism.[47]

The suppressive effect of genistein on osteoclasts was not altered in the presence of tamoxifen, an antagonist of estrogen, which did not have an appreciable effect on the cells.[47] The effect of genistein may be not based primarily on the mode of estrogen action on osteoclasts.

Effect of Isoflavone on Osteoclast Function

Osteoclasts were isolated from rat femoral tissues. The effect of genistein or calcium in decreasing the number of osteoclasts was significantly blocked in the presence of magnesium, although the effect of isoflavone was not appreciably altered by zinc or vanadate.[54] Magnesium has been shown to inhibit the Ca^{2+} channel in the plasma membranes of osteoclasts.[55] Zinc can produce an activation of Ca^{2+} sensor in the cells and it may induce an increase in intracellular Ca^{2+} levels.[56] These observations may support the view that the suppressive effect of genistein on osteoclasts is induced through Ca^{2+} signaling in the cells.[48] However, the effect of genistein was partly blocked by magnesium, whereas the suppressive effect of calcium on osteoclasts was completely prevented by magnesium.[54] This finding sug-
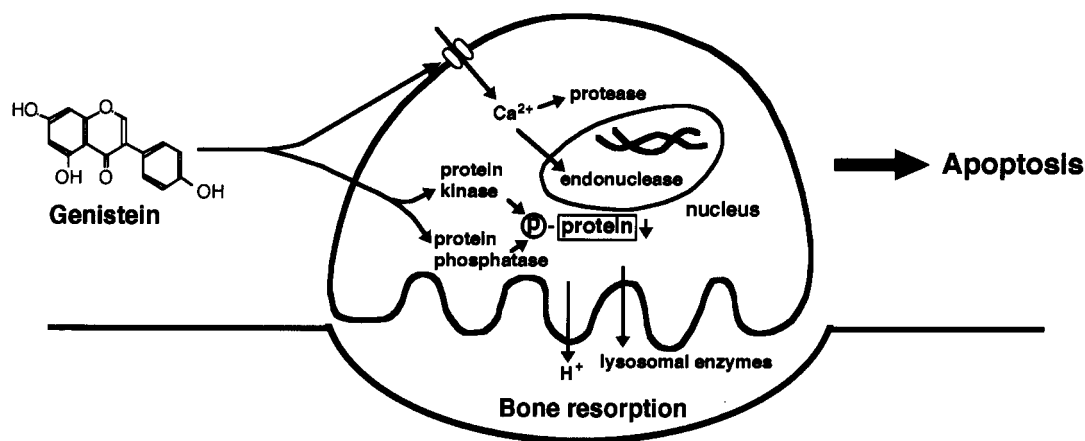
Fig. 5. Cellular Mechanism by Which Isoflavone Inhibits Osteoclastic Bone Resorption

    Genistein stimulates Ca^{2+} entry into osteoclasts and induces apoptosis related to Ca^{2+} signaling. The isoflavone inhibits protein kinases and activates protein phosphatases in osteoclasts, thereby inhibiting osteoclastic bone resorption.

gests that the suppressive effect of genistein on osteoclasts is partly mediated through other mechanisms in addition to Ca^{2+} signaling mechanisms.[54]

    The culture of osteoclasts with genistein caused a significant inhibition of protein kinase activity and an appreciable elevation of protein tyrosine phosphatase activity.[54] Genistein has been shown to have a strong inhibitory effect on protein tyrosine kinases.[6,7] The inhibition of these kinases may induce apoptosis in a human ovarian tumor cell line[6] and in Jurket T-leukemia cells.[7] Genistein may partly induce apoptosis of osteoclasts by inhibiting protein tyrosine kinases in the cells. It has been shown that tyrosine kinase Src is implicated in the process of osteoclast-induced bone resorption *in vitro* and *in vivo*.[57]

    The culture with genistein of osteoclasts caused a significant increase in protein tyrosine phosphatase activity.[54] Such an effect was also seen by the addition of genistein (10^{-7}–10^{-5} M) in the enzyme reaction mixture *in vitro*.[54] This finding that genistein can directly activate protein tyrosine phosphatase in osteoclasts is reported for the first time to our knowledge. Vanadate is an inhibitor of protein tyrosine phosphatase.[57] The culture of osteoclasts with vanadate did not cause a decrease in the number of osteoclasts. The suppressive effect of genistein on osteoclasts was also seen in the presence of vanadate.[54] If the phosphorylation of proteins in osteoclasts is partly involved in osteoclastic bone resorption,[58] the activation of protein tyrosine phosphatase may participate to some degree in the inhibition of bone resorption through the reduction of phosphorylated protein levels. It has been reported that protein ty-

rosine phosphatase (Src homology 2 domain-containing tyrosine phosphatase 1) is a negative regulator of osteoclastogenesis and osteoclast resorbing activity in mutant mice.[59] Presumably, the suppressive effect of genistein on osteoclasts is partly mediated through an activation of protein tyrosine phosphatase in the cells.

    β-Glucuronidase is a lysosomal enzyme which is related to stimulation of bone resorption by PTH.[36] Genistein did not have an effect on β-glucuronidase activity in osteoclasts.[54] The effect of genistein on inhibition of osteoclastic bone resorption may not be implicated in the activity of lysosomal enzymes in the cells.

    In conclusion, the suppressive effect of genistein on rat bone osteoclasts may be involved in the induction of apoptosis which is mediated through Ca^{2+} signaling mechanism, the inhibition of protein kinase and the activation of protein tyrosine phosphatase in the cells, as shown in Fig. 5.

PREVENTIVE EFFECT OF ISOFLAVONE ON BONE LOSS

Preventive Effect of Dietary Isoflavone on Bone Loss *in Vivo*

    Bone mass decreases with age.[12–16] This decrease may be due to increased bone resorption and to decreased bone formation. Osteoporosis with decrease of bone mass is widely recognized as a major public health problem. Pharmacological and nutritional factors have the potential to prevent bone loss with increasing age, however, these factors are not yet fully
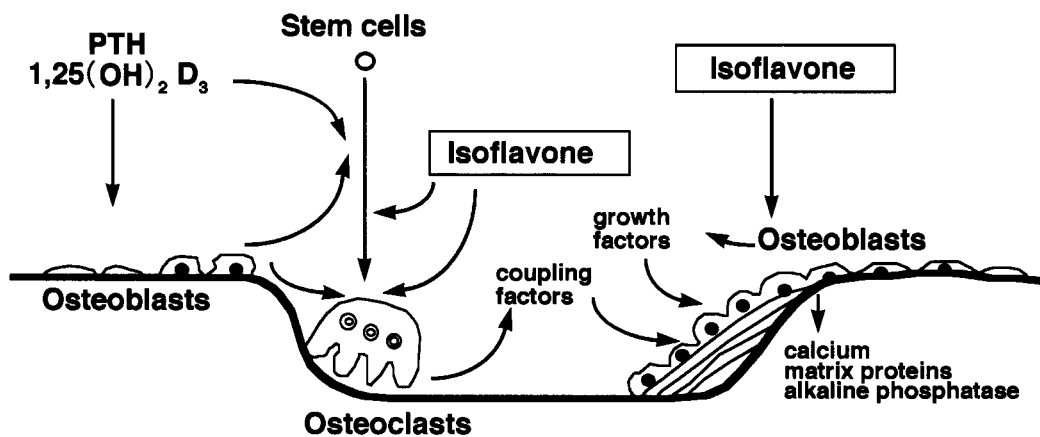
Fig. 6. Action of Isoflavone to Prevent Bone Loss

    Genistein and daidzein act on osteoblastic cells and stimulate the production of bone matrix protein components from the cells. Also, these isoflavones prevent the bone-resorbing factors-induced osteoclast-like cell formation from marrow cells and induce apoptosis of osteoclasts, which inhibits osteoclastic bone resorption.

understood. Genistein and daidzein have been demonstrated to stimulate osteoblastic bone formation[9–11,21–23] and to inhibit osteoclastic bone resorption,[33,41,47,54] so that bone mass is increased, as shown in Fig. 6. Isoflavones may have potential to prevent bone loss with increasing age. In fact, genistein has been reported to inhibit bone loss in ovariectomized rats,[60,61] an animal model of osteoporosis.

    Isoflavones including daidzin, daidzein, genistin, and genistein are present in soybean in great quantities. Daidzin and genistin are hydrolyzed to daidzein and genistein by β-glucosidase in the gastric intestine, respectively (Fig. 1). *Nijiru*, which is a by-product in the fermentation process of soybean to make *natto*, contains great quantities of isoflavones. *Nijiru* contains a natural isoflavone and is a functional food factor as a dietary natural isoflavone. The oral administration of this isoflavone-containing soybean extract (*nijiru*) to rats caused a significant increase in bone components (calcium, alkaline phosphatase, and DNA) in the femoral-diaphyseal and -metaphyseal tissues *in vivo*,[62,63] indicating its anabolic effect on bone metabolism.

    Ovarian hormone deficiency at menopause stimulates bone loss.[64,65] Ovariectomy (OVX) causes a lack of estrogen and it has been established that estrogen deficiency induces osteoporosis in humans and in rats.[66] In fact, bone weight, bone mineral density and bone mineral content are reduced in OVX rats.[67] These reductions were significantly prevented by the feeding of dietary fermented soybean with supplementation of *nijiru* containing isoflavones for 3 months.[67] This finding indicates that OVX-induced bone loss can be prevented by the prolonged intake

of dietary isoflavone supplementation.

    Twelve volunteers (six men and six women) received *nijiru* twice a day for 60 days at a dose of 1500 mg (6 tablets) per day. Serum γ-carboxylated osteocalcin concentration was significantly increased by the intake of *nijiru* in both sexes to about 2-fold that in the control group.[68] Liver and renal function was not affected by *nijiru* supplementation. The intake of isoflavone-containing *nijiru* can stimulate the γ-carboxylation of osteocalcin, which plays an important role in bone formation and mineralization,[69] in healthy individuals. Thus the intake of isoflavone-containing *nijiru* as a supplement may have a role in the prevention of age-related bone loss.

Synergistic Effect of Isoflavone and Zinc on Bone Metabolism

    Whether the combination of nutritional factors has an additive or synergistic effect on bone components has not been determined, and this knowledge may be important in the prevention of bone loss with age. Recently, it has been shown that the combination of genistein and zinc can have a synergistic effect on bone components in femoral tissue from elderly female rats *in vitro* and *in vivo*.[70–72] Incidentally, zinc, an essential trace element, has been demonstrated to have a potent stimulatory effect on bone formation and an inhibitory effect on bone resorption, supporting its preventive effect on bone loss with aging[73–75] (in Review).

    The mechanism by which the combination of genistein and zinc reveals a synergistic effect in increasing bone alkaline phosphatase activity, DNA, and calcium content in bone tissue culture *in vitro* is

unknown. The effect of the combination of genistein and zinc in elevating bone components, however, is completely abolished in the presence of cyclohex-imide, an inhibitor of protein synthesis, in a tissue culture system *in vitro*,[71] suggesting that this effect is based on a newly synthesized protein component. Genistein has been reported to bind the estrogen receptor-β on bone cells.[24] It is possible that genistein binds to the strogen receptors and that the isoflavone may be exerting effects similar to those of estrogen in osteoblasts. Zinc can stimulate protein synthesis in osteoblastic cells.[73,75] The gene sequence for the receptor for the steroid hormone has been shown to have zinc fingers at the site of interaction with DNA.[76] The effect by the combination of genistein and zinc may be partly based on the stimulation of synthesis of estrogen receptor proteins or the potentiation of the interaction of the estrogen-receptor complex with DNA at that site in osteoblastic cells. In addition, zinc may stimulate promoter activity directly.

The effect of experimental diets with fermented soybean containing genistein and zinc on OVX-induced bone loss has been demonstrated.[77] Experimental diets contained 2.1 to 9.7 mg zinc per 100 g of diet and 44.6 to 92.4 mg isoflavone (including genistin, genistein, daidzin, and daidzein) per 100 g of diet was fed freely to OVX rats for 3 months. OVX caused a significant reduction in the dry weight, mineral density, calcium content, zinc content and alkaline phosphatase activity in femoral tissues.[77] The reductions were largely prevented by feeding a *natto* diet. This prevention was significantly enhanced in OVX rats fed a *natto* diet supplemented with isoflavone and zinc.[77] The prolonged intake of dietary *natto* supplemented with isoflavone and zinc has a preventive effect on OVX-induced bone loss, suggesting that it may have a role in the prevention of osteoporosis.

Synergistic Effect of Isoflavone and Casein Phosphopeptides on Bone Metabolism

Casein phosphopeptides (CPP), a product of tryptic casein digestion, have been shown to enhance paracellular transport of calcium in the distal small intestine.[78] CPP have been reported to prevent the precipitation of insoluble calcium phosphate salts by forming soluble complexes with ionized calcium *in vitro* and *in vivo* in intestinal luminal contents, thereby promoting the passive absorption of calcium in the ileum.[79,80] CPP-containing diets have been shown to have a preventive effect on OVX-induced

bone loss in rats.[81]

Genistein (10 or 50 μg/100 g body weight) or CPP (40 mg/100 g) was orally administered to young (5 weeks old) or elderly (50 weeks old) female rats for 14 days. The administration of genistein (50 μg/100 g) resulted in a significant increase in calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and -metaphyseal tissues of young and elderly rats.[82] The administration of CPP (40 mg/100 g) caused a significant increase in the femoral dry weight of both rat age groups. CPP administration increased significantly the diaphyseal calcium content in young rats, but had no effect on the diaphyseal or metaphyseal alkaline phosphatase activity or DNA content.[82] The genistein (50 μg/100 g)-increased femoral dry weight and bone components were significantly enhanced by the simultaneous administration of CPP (40 mg/100 g).[82] The combination of genistein and CPP administration had a synergistic-anabolic effect on bone components in rats with increasing age. This effect may be from the stimulatory action of genistein on osteoblastic cells, which are related to bone formation and mineralization, and the enhancing effect of CPP on intestinal calcium absorption, by exhibiting different mechanisms. The combination of genistein and CPP in dietary supplementation may be a good tool in the prevention of bone loss with aging.

PROSPECTS

Soybeans contain great quantities of saponin and glycitein in addition to genistin, genistein, daidzin, and daidzein.[63] Genistin and daidzin are hydrolyzed to genistein and daidzein by β-glucosidase in the gastrointestinal system, respectively. Saponin and daidzein had an anabolic effect on bone metabolism.[11,14] Genistein, however, had a more potent effect on bone calcification than saponin[63] or daidzein.[11] Glycitein had no effect on bone calcification.[20] Resveratol, catechin, and (-1)-epigallocatechin gallate, which are polyphenols in food, do not have an anabolic effect on bone calcification.[20] A phytoestrogen genistein has a unique anabolic effect on bone metabolism.

Genistein is a 4′,5,7-trihydroxyisoflavone, and daidzein is a 4′,7-dihydroxyisoflavone. Glycitein is a 4′, 7-dihydroxy-6-methoxyisoflavone, while quercetin is a 3,3′,4′,5,7-pentahydroxyflavone; neither has any effect on bone calcification *in vitro*. The ana-

bolic effect of genistein and daidzein on bone calcification is weakened by the phosphorylation of the hydroxy-group at their 7-position.[19] The hydroxy-groups at the 5- and 7-positions of the isoflavone genistein may be necessary for its anabolic effect on bone calcification, suggesting a chemical structure-activity relationship.

The action of genistein in stimulating osteoblastic bone formation and inhibiting osteoclastic bone resorption is based on a non-genomic effect and genomic effect which are involved in estrogen receptors. Genistein can directly inhibit protein kinases[6,7,54] and activate protein phosphatase[54] both of which are implicated in the intracellular signaling mechanism in cells. In addition, genistein may be able to enhance promoter activity which is not related to estrogen receptors-DNA binding in the nucleus of cells. The molecular mechanism of genistein action remains to be determined.

The intake of soybean foods containing isoflavones may have a preventive effect on bone loss with increasing age. Isoflavone may be important as a food factor in maintaining healthy bone and the supplemental intake of dietary isoflavone may play a role in the prevention of osteoporosis in otherwise healthy individuals.

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