—Reviews—

Regulatory Mechanism of Food Factors in Bone Metabolism and Prevention of Osteoporosis

Masayoshi YAMAGUCHI

Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52–1 Yada, Suruga-ku, Shizuoka City 422–8526, Japan

(Received June 27, 2006)

Aging induces a decrease in bone mass, and osteoporosis with its accompanying decrease in bone mass is widely recognized as a major public health problem. Bone loss with increasing age may be due to decreased bone formation and increased bone resorption. Pharmacologic and nutritional factors may prevent bone loss with aging, although chemical compounds in food and plants which act on bone metabolism are poorly understood. We have found that isoflavones (including genistein and daidzein), which are contained in soybeans, have a stimulatory effect on osteoblastic bone formation and an inhibitory effect on osteoclastic bone resorption, thereby increasing bone mass. Menaquinone-7, an analogue of vitamin K_2 which is abundant in fermented soybeans, has been demonstrated to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption. Of various carotenoids, β-cryptoxanthin, which is abundant in Satsuma mandarin (*Citrus unchiu* MARC), has a stimulatory effect on osteoblastic bone formation and an inhibitory effect on osteoclastic bone resorption. The supplementation of these factors has a preventive effect on bone loss induced by ovariectomy in rats, which are an animal model of osteoporosis, and their intake has been shown to have a stimulatory effect on bone mass in humans. Factors with an anabolic effect on bone metabolism were found in extracts obtained from wasabi leafstalk (*Wasabi japonica* MATSUM), the marine alga *Sargassum horneri*, and bee pollen *Cistus ladaniferus*. Phytocomponent *p*-hydroxycinnamic acid was also found to have an anabolic effect on bone metabolism. Food chemical factors thus play a role in bone health and may be important in the prevention of bone loss with increasing age.

Key words——isoflavone; menaquinone-7; β-cryptoxanthin; *p*-hydroxycinnamic acid; bone metabolism; osteoporosis

INTRODUCTION

Aging induces a decrease in bone mass. Osteoporosis with its accompanying decrease in bone mass is widely recognized as a major public health problem. The most dramatic expression of the disease is represented by fractures of the proximal femur, the number of which increases as the population ages.[1—5] 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.[6,7] Deficiency in both micronutrients and macronutrients appears to be strongly implicated in the pathogenesis and the consequences of hip fracture in the osteoporotic elderly.[8] Nutritional and pharmacologic factors are important in preventing bone loss with increasing age.

Chemical compounds in food and plants which act on bone metabolism have not been fully clarified. Recent studies have shown that isoflavones, which are contained in soybeans, menaquinone-7 (vitamin K_2), which is abundant in fermented soybeans, and β-cryptoxanthin, which is present in fruit and vegetables, have been demonstrated to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption *in vitro*. The supplementation of isoflavones, menaquinone-7, and β-cryptoxanthin has preventive effects on bone loss induced by ovariectomy in rats, which is an animal model of osteoporosis, and their intake has a stimulatory effect on bone mass in humans. Food chemical factors thus play a role in bone health and may be important in the prevention of bone loss with aging.

This review mainly describes our findings concerning the biochemical action of food factors on bone metabolism and their preventive effects on osteoporosis.

ISOFLAVONE AND BONE METABOLISM

Isoflavones including daidzin, daidzein, genistin, and genistein are present in soybeans at relatively high concentrations. As shown in Fig. 1, daidzin and genistin are hydrolyzed to daidzein and genistein by

e-mail: yamaguch@u-shizuoka-ken.ac.jp

This review was described as a commemoration for receiving of Senji Miyata Foundation Award 2005.

β-glucosidase in the gastrointestinal system, respectively. Genistein has been shown to have a strong inhibitory effect on protein tyrosine kinase and it can cause cell cycle arrest and apoptosis in leukemic cells. Such a cellular mechanism may be important in the prevention of cancers. We demonstrated that genistein and daidzein have an anabolic effect on bone metabolism in rats, suggesting their role in the prevention of osteoporosis, which is widely recognized as a major public health problem. Isoflavone as a food factor is useful in the prevention of and therapy for osteoporosis.

Action of Isoflavone on Bone Formation in Tissue Culture Bone metabolism is regulated by osteoblasts and osteoclasts localized in bone tissues. Osteoblasts stimulate bone formation and calcification, while osteoclasts promote bone resorption. The anabolic effects of genistein on bone metabolism have been investigated in tissue culture using the femoral-metaphyseal (trabecular bone) tissues obtained from elderly female rats *in vitro*.[9,10]

Bone tissues were cultured for 24 h in Dulbecco's modified Eagle's medium (MEM) (high glucose, 4.5 %) with bovine serum albumin (serum free) and antibiotics containing either vehicle or genistein. The presence of genistein (10^{-6} or 10^{-5} M) was found to induce a significant increase in calcium content, alkaline phosphatase activity, which is a marker enzyme of osteoblasts, and DNA content, which is an index of bone cell number in bone tissues, in rat femoral-metaphyseal (trabecular bone) tissues.[9] The effects of genistein in increasing bone components were equal to the stimulatory effects of 17β-estradiol, which is physiologically important. Anti-estrogen agent tamoxifen was shown to inhibit completely the genistein-induced increase in bone components, although tamoxifen itself had no effect on bone components.[9] The anabolic effects of genistein on bone metabolism may be partly mediated through estrogen-like action. Presumably, genistein binds to the receptor of estrogen in osteoblastic cells in which this receptor is localized.[11,12]

The effects of genistein or genistin on bone components were compared in an *in vitro* culture system.[10] Culture with genistein (10^{-8}—10^{-5} M) or genistin (10^{-7}—10^{-5} M) was shown to increase alkaline phosphatase activity and DNA and calcium contents in the femoral-metaphyseal tissues obtained from elderly female rats.[10] The effects of genistein or genistin 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 effects of either isoflavone are partly based on newly synthesized protein components.

The effects of daidzein on the femoral-diaphyseal (cortical bone) tissues obtained from elderly female rats *in vitro* were also investigated.[13] The presence of

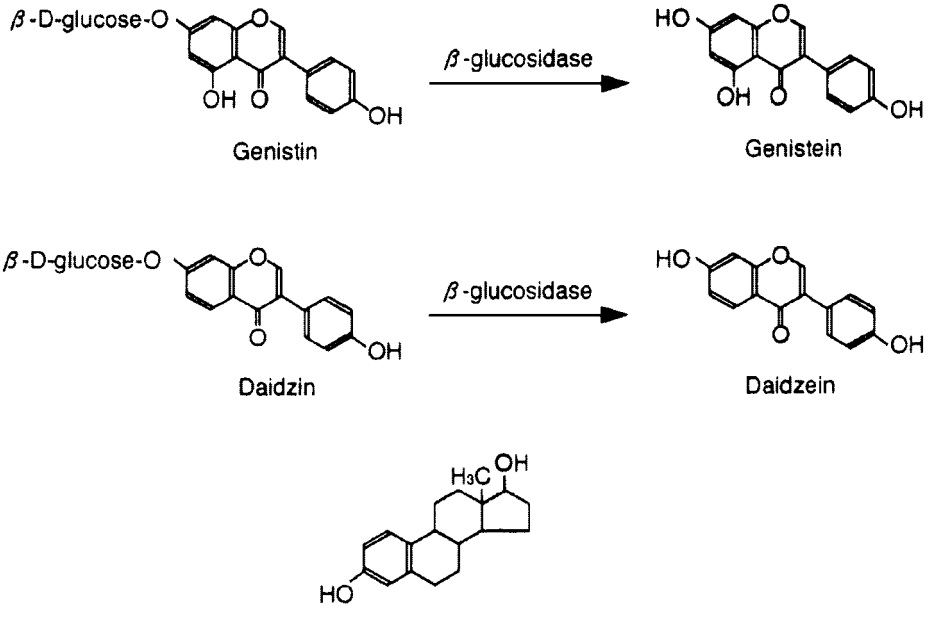

Fig. 1. Chemical Structure of Isoflavones

daidzein (10^{-6}—10^{-5} M) in culture medium caused a significant increase in bone components. This effect was equal to that of genistein (10^{-6} or 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 suppressed in the presence of cycloheximide. Genistein is a 5, 7, 4′-trihydroxyisoflavone, and daidzein is a 7,4-dihydroxyisoflavone, and the chemical structure of the two compounds is similar. Presumably, the chemical form of dihydroxyisoflavone is important in its anabolic effects on bone. Genistein and daidzein may have a bone effect that operates via the same mechanism.

The effects of phosphogenistein and phosphodaidzein, which are phosphorylated for the hydroxyl group (OH) at the 7-position of genistein and daidzein, on bone components have been demonstrated.[14] Phosphogenistein or phosphodaidzein increases bone components in tissue culture *in vitro*. Phosphoisoflavones at a lower concentration of 10^{-6} M, at which genistein and daidzein have anabolic effects on bone components, had no effect.

The effects of various polyphenols found in food and plants on bone metabolism have been determined. Glycitein, resveratol, quercetin, catechin, and (−)-epigallocatechin gallate do not have anabolic effects on bone calcification *in vitro*.[15] Polyphenols do not directly stimulate bone calcification *in vitro*. Genistein has unique anabolic effects on bone.

Genistein is a 4′, 5, 7-trihydroxyisoflavone, and daidzein is a 4′, 7-dihydroxyisoflavone. Glycitein is a 4′, 7-dihydroxy-6-methoxyflavone, while quercetin is a 3,3′,4′,5,7-pontahydeoxyflavone; neither has any effect on bone calcification *in vitro*. The anabolic effect of genistein and daidzen on bone calcification is weakened by the phosphorylation of the hydroxy-group at their-7-position.[14] The hydroxy-groups at the 5- and 7-positions of the isoflavone genistein may be necessary for its anabolic effect on bone formation, suggesting a chemical structure-activity relationship.

Effects of Isoflavones on Osteoblastic Cells Osteoblasts play a role in the stimulation of bone formation and calcification. The action of isoflavones on osteoblastic cells *in vitro* is shown.[16—18] Osteoblastic MC3T3-E1 cells were cultured for 48 h in a serum-free α-MEM containing genistein or daidzein. The presence of genistein (10^{-6} or 10^{-5} M) or daidzein (10^{-6} or 10^{-5} M) caused a significant increase in protein content, alkaline phosphatase activity, and DNA content in the cells. The ability of genistein or daidzein to increase biochemical components in the cells is not seen in the presence of cycloheximide, suggesting that the effects of isoflavone result from newly synthesized protein components. The anabolic effects of genistein in osteoblastic cells are not distinguishable from those of daidzein.

The effects of 17β-estradiol in increasing protein content and alkaline phosphatase activity in osteoblastic cells is not enhanced in the presence of genistein.[16] Cellular protein content was additively increased with 17β-estradiol and daidzein, but their effects on alkaline phosphatase activity were not additive. The receptors of estrogen are found in osteoblastic cells.[11,12] Genistein has been shown to bind to estrogen receptor β in osteoblastic cells.[19] It is speculated that genistein or daidzein may bind to estrogen receptor β in osteoblastic cells, although the nuclear localization of genistein or daidzein in these cells has not been fully clarified.

DNA content in osteoblastic cells was significantly increased in the presence of genistein or daizein, suggesting that isoflavones stimulate cell proliferation.[16,17] Isoflavones increase alkaline phosphatase activity, which is a marker enzyme in the differentiation of osteoblastic cells. Genistein and daidzein may have stimulatory effects on the proliferation and differentiation of osteoblastic MC3T3-E1 cells.

The cellular mechanism by which isoflavones stimulate osteoblastic bone formation has been studied in relation to protein synthesis. The presence of genistein or daidzein stimulates protein synthesis in osteoblastic MC3T3-E1 cells *in vitro*.[18] The addition of genistein (10^{-7}—10^{-5} M) or daidzein (10^{-7}—10^{-5} M) to the reaction mixture of protein synthesis using the cell homogenate from osteoblastic cells cultured without isoflavone caused a significant increase in protein synthesis *in vitro*. This increase is clearly suppressed 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 [^{3}H]leucyl-tRNA synthetase activity in the cytosol fraction of osteoblastic cell homogenate.[18] Genistein had a greater effect than daidzein. Isoflavone can directly activate leucyl-tRNA synthetase, a rate-limiting enzyme in the translational process of protein synthesis.[20]

The possibility cannot be excluded that genistein or daidzein acts on the translational process in osteoblastic MC3T3-E1 cells, since the stimulatory effects of the isoflavones on protein synthesis in osteoblastic cells are suppressed by actinomycin D. Genistein has been shown to bind to estrogen receptors in osteoblastic cells,[21] although daidzein cannot bind to estrogen receptors.[22] Moreover, it is possible that genistein or daidzein can bind to transcriptional proteins, which differ from estrogen receptors, in osteoblastic MC3T3-E1 cells. Whether isoflavones have effects on various protein kinases and protein phosphatases that are related to osteoblastic cell proliferation and nuclear transcription activity must be determined.

As mentioned above, genistein and daidzein have anabolic effects in osteoblastic MC3T3-E1 cells. Isoflavones may stimulate osteoblastic bone formation, supporting the observation that they have an overall stimulatory effect on bone formation and mineralization in bone tissue culture systems.

Effects of Isoflavones on Bone Resorption in Tissue Culture Parathyroid hormone (PTH), prostaglandin E_2, (PGE_2), and lipopolysaccharide (LPS) have stimulatory effects on bone resorption in *in vitro* culture system.[23—26] Culture with PTH, PGE_2, or LPS clearly stimulated bone resorption in the femoral-metaphyseal tissues cultured for 48 h, when bone resorption was estimated with a decrease in bone calcium content.[27] The effects of bone-resorbing factors were completely inhibited in the presence of genistein, indicating inhibition of bone resorption.[27]

PTH and PGE_2 cause a remarkable increase in glucose consumption and lactic acid production by bone tissues.[27] The production of lactic acid by 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. Genistein completely inhibited the PTH- or PGE_2-induced increase in both glucose consumption and lactic acid production by bone tissues.[27] The inhibitory effects of genistein on bone resorption are partly related to the prevention of lactic acid production by bone tissues.

Genistein has stimulatory effects on bone formation and mineralization in tissue culture *in vitro*. The isoflavone can also inhibit bone resorption in tissue culture. Genistein may thus have an important role in the preservation of bone mass.

Effects of Isoflavones on Mature Osteoclastic Cells Osteoclasts, or bone-resorbing cells, are formed from bone marrow.[28] Osteoclast-like cell formation is estimated based on staining for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts.[29,30] Mouse bone marrow cells were cultured for 7 days in medium containing a bone-resorbing agent [PTH, PGE_2, 1,25-dihydroxyvitamin D_3 (VD_3), or LPS] at an effective concentration.[31] Culture with PTH, PGE_2, VD_3, or LPS induced a remarkable increase in osteoclast-like multinucleated cells. These increases were significantly inhibited in the presence of genistein.[31] The inhibitory effects of genistein (10^{-5} M) were equal to the effects of other anti-bone-resorbing agents (calcitonin, 17β-estradiol, and zinc sulfate) on osteoclast-like cell formation in mouse marrow culture.[31] Genistein had potent inhibitory effects at the later stage of differentiation of marrow cells. The inhibitory effects of genistein on osteoclast-like MNC formation in mouse marrow culture were greater than that of daidzein. The inhibitory effect of genistein on osteoclast-like MNC formation induced by PTH or PGE_2 may be partly based on the blocking of the pathway of cyclic AMP signaling, but not that by protein kinase C signaling, at the differentiation stage of marrow cells. In addition, if genistein can inhibit tyrosine kinase,[32] the action of isoflavones on osteoclastic cell formation may be related in part to inhibitory effects on the kinase. The cellular mechanism by which genistein inhibits osteoclast-like cell formation from marrow cells may be involved in cyclic AMP signaling.

Genistein was found to induce cell death (apoptosis) of osteoclasts isolated from rat femoral tissues.[33] Calcitonin is known to inhibit osteoclast activity.[34] The hormonal effect may be mediated through the two pathways of cyclic AMP and Ca^{2+} signalings.[35] The addition of calcitonin, DcAMP, or calcium chloride to culture medium suppressed the number of osteoclasts isolated from rat femoral tissues.[33] The addition of dibucaine, an antagonist of calmodulin, or staurosporine, an inhibitor of protein kinase C, completely prevented the decrease in osteoclasts induced by calcitonin, DcAMP, or calcium chloride.[33] The effect of genistein in decreasing osteoclasts was clearly suppressed by these inhibitors, suggesting that the effects of isoflavones on mature osteoclasts is partly mediated through the pathway of Ca^{2+} signaling.

A Ca^{2+} ionophore (A2387) caused a remarkable decrease in the number of osteoclasts, indicating that

the entry of Ca^{2+} in the cells induces osteoclast death.[33] The number of osteoclasts was decreased when cultured with genistein or calcium chloride. Ca^{2+} can activate endonuclease and the metal induces apoptosis in cells.[36] 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 torosiomerase II.[37] 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. In addition, daidzein has also been shown to suppress the number of mature osteoclasts,[33] although daidzein does not have a greater suppressive effect than genistein. The effect of daidzein is completely abolished in the presence of dicucaine or staurosporine, supporting that the partial involvement of the Ca^{2+}-signaling mechanism.[33]

Culture with genistein is found to induce a significant inhibition of protein kinase activity and an appreciable elevation of protein tyrosine phosphatase activity in osteoclasts.[38] Genistein has a strong inhibitory effect on protein tyrosine kinases.[32] The inhibition of these kinases may induce apoptosis in a human ovarian tumor cell line[32] and in Jurket T-leukemia cells.[39] Genistein may partly induce apoptosis of osteoclasts through a mechanism that inhibits 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*.[40]

Culture with genistein is found to cause a significant increase in protein tyrosine phosphatase activity in osteoclasts. Such an effect is also seen with the addition of genistein to the enzyme reaction mixture *in vitro*. Genistein can directly activate protein tyrosine phosphatase in osteoclasts. Vanadate is an inhibitor of protein tyrosine phosphatase. The culture of osteoclasts with vanadate does not cause a decrease in the number of osteoclasts. The suppressive effect of genistein on osteoclasts is also seen in the presence of vanadate.[38] It has been reported that protein tyrosine phosphatase (Src homology 2 domain-containing tyrosine phosphatase) is a negative regulator of osteoclastogenesis and osteoclast-resorbing activity in mutant mice.[40] Presumably, the suppressive effect of genistein on mature osteoclasts is partly mediated through the activation of protein tyrosine phosphatase in the cells.

β-Glucuronidase is a lysosomal enzyme involved in stimulation of bone resorption by PTH. Genistein does not have an effect on β-glucuronidase activity in osteoclasts.[38] The effects 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 effects of genistein on rat bone osteoclasts may be involved in the induction of apoptosis mediated through the Ca^{2+} signaling mechanism, inhibition of protein kinase, and activation of protein tyrosine phosphatase in the cells.

Preventive Effects of Isoflavones against Bone Loss

Bone mass decreases with age. This decrease may be due to increased bone resorption and to decreased bone formation. Osteoporosis with a decrease in bone mass is widely recognized as a major public health problem. Genistein and daidzein have been demonstrated to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption, so that bone mass is increased, as shown in Fig. 2. Isoflavones may have the potential to prevent bone loss with increasing age.

Nijiru, which is a by-product in the fermentation process of soybeans to make *natto*, contains large quantities of natural isoflavone. *Nijiru* is a functional food factor as a dietary isoflavones. The oral administration of the 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*,[41,42] indicating its anabolic effects on bone metabolism.

Ovarian hormone deficiency at menopause stimulates bone loss.[43,44] Ovariectomy (OVX) causes a lack of estrogen, and the hormone deficiency induces osteoporosis in humans. Bone weight, bone mineral density, and bone mineral content are reduced in OVX rats.[45] These reductions were significantly prevented by feeding with fermented soybeans with *nijiru* supplementation containing isoflavones for 3 months,[45] indicating that OVX-induced bone loss can be prevented by the prolonged intake of dietary isoflavone supplementation.

A supplement made from *Nijiru* powder was shown to enhance bone healthy in individuals. Twelve volun-

teers (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 with the intake of *nijiru* in both sexes to about 2-fold that in the control group.[46] The intake of isoflavone-containing *nijiru* as a supplement may thus have a role in the prevention of age-related bone loss.

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. The combination of genistein and zinc was found to have a synergistic effect on bone components in femoral tissue from elderly female rats *in vitro* and *in vivo*.[47—49] 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.[50—52]

The effects of experimental diets with fermented soybeans containing genistein and zinc on OVX-induced bone loss has been demonstrated.[53] Experimental diets containing 2.1 to 9.7 mg of zinc per 100 g of diet and 44.6 to 92.4 mg of isoflavones (including genistein, genistin, daidzin, and daidzein) per 100 g of diet was fed 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.[53] 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. 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.

It has also been shown that the intake of zinc-supplemented *natto* has a potent stimulatory effect on bone formation and an inhibitory effect on bone resorption in aged individuals.[54] The prolonged intake of dietary isoflavone-rich *natto* supplemented with zinc may be useful for bone health and the prevention of bone loss.

Casein phosphopeptides (CPP), a product of tryptic casein digestion, have been shown to enhance paracellular transport of calcium in the distal small intestine.[55,56] Genistein or CPP was orally administered to young (5 weeks old) or elderly (50 weeks old) female rats for 14 days.[57,58] The genistein-induced increase in femoral dry weight and bone components was significantly enhanced by the simultaneous administration of CPP.[58] The combination of genistein and CPP administration has an synergistic anabolic effect on bone components in rats with increasing age. The combination of genistein and CPP in dietary supplementation may be a good tool for the prevention of bone loss with aging.

MENAQUINONE-7 AND BONE METABOLISM

Vitamin K_2 is essential for the γ-carboxylation of osteocalcin, a bone matrix protein containing γ-carboxyglutamic acid, which is synthesized only in osteoblasts.[59,60] Noncarboxylated osteocalcin cannot bind to hydroxyapatite in bone tissues.[60,61] Much attention has been paid to the role of vitamin K in bone metabolism. The chemical structure of vitamin K is shown in Fig. 3. There are three types of vitamin K: vitamin K_1 (phylloquinone), vitamin K_2 (menaquinone), and vitamin K_3 (menadione). Vitamin K_1 is a sole compound, but vitamin K_2 is a series of vitamers with multiisoprene units (1 to 4) at the 3-position of the naphthoquinone. Several reports have indicated the effects of vitamin K_1 on bone metabolism.[62,63] In contrast, the effects of vitamin K_2 on bone metabolism have not attracted notice. Like vitamin K_1, vitamin K_2 (menatetrenone), with four isoprene units, not only enhances mineralization but also increases the amount of osteocalcin in cultured human osteoblasts.[64] It has also been reported that menatetrenone inhibits bone resorption, which may be related to its side chain[65] and that the compound inhibits bone loss induced by ovariectomy in rats.[66]

Natural vitamin K_2 (menaquinone-7; MK-7) with seven isoprene units is abundant in fermented soybeans (*natto*). The effects of MK-7 on bone metabolism have not been clarified. We found that MK-7 has an anabolic effect on bone calcification in rat femoral tissues.[67] MK-7 may play a role in the prevention of bone loss with increasing age.

Effects of MK-7 on Osteoblastic Bone Formation MK-7 has been found to have a stimulatory effect on calcification in the femoral tissues obtained from normal young rats *in vitro*.[67] Culture with MK-7 (10^{-6} or 10^{-5} M) caused a significant increase in biochemical components (calcium content, alkaline phosphatase activity, and DNA content) in the femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone)

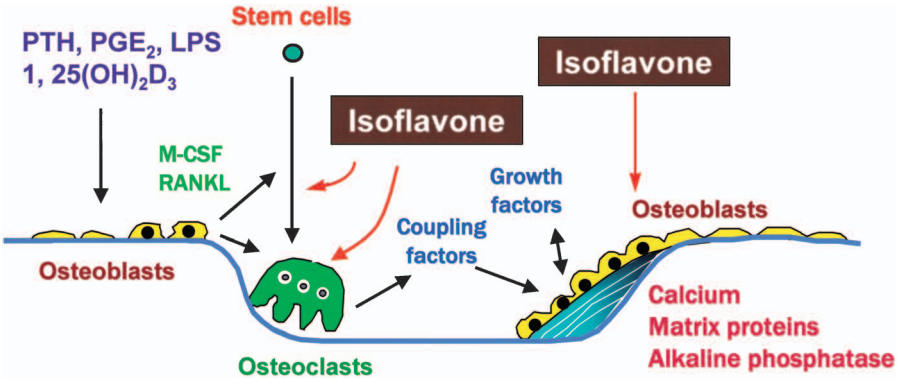

Fig. 2. Action of Isoflavones to Prevent Bone Loss

Isoflavones (including genistein or daidzein) have a stimulatory effect on osteoblastic bone formation and an inhibitory effect on osteoclastic bone resorption, thereby increasing bone mass.

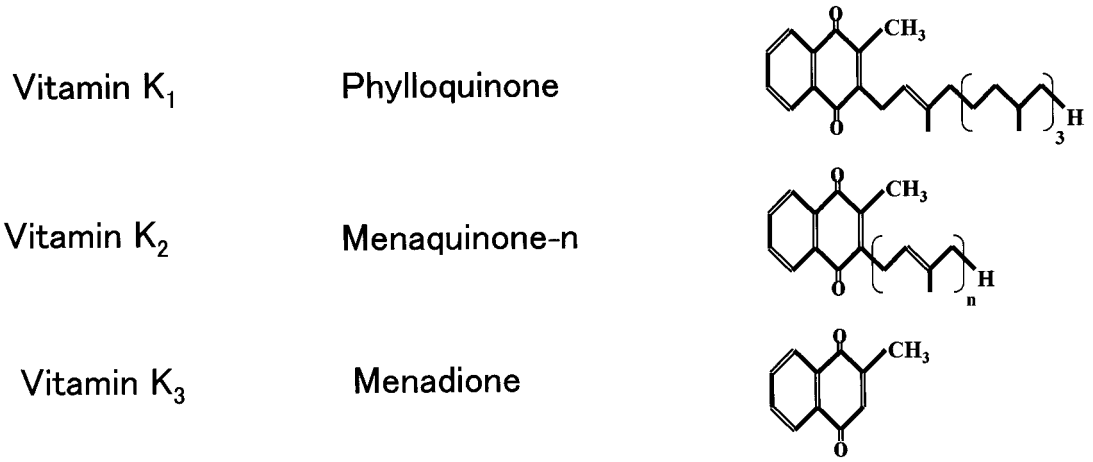

Fig. 3. Chemical Structure of Vitamin K

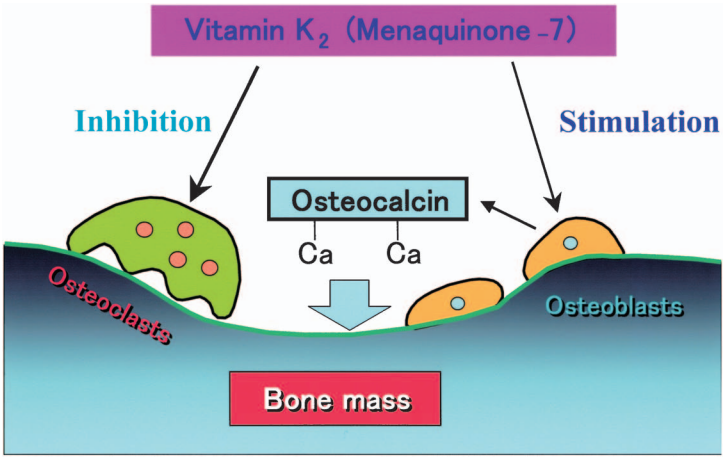

Fig. 4. Action of Menaquinone-7 in Increasing Bone Mass

Menaquinone-7 stimulates protein synthesis in osteoblastic cells and enhances osteocalcin production, a calcium-binding protein, in the cells. This protein stimulates mineralization in bone tissues. Menaquinone-7 also has an inhibitory effect on osteoclastic bone resorption.

tissues obtained from aged rats *in vitro*.[68] This effect was significantly enhanced in the presence of genistein (10^{-6} or 10^{-5} M), suggesting that the mode of action of MK-7 differs from that of genistein. The effect of MK-7 in increasing bone components in the femoral tissues was completely abolished in the presence of cycloheximide, an inhibitor of protein synthesis *in vitro*. MK-7 has a stimulatory effect on bone formation in the femoral tissues of elderly female rats *in vitro*.

Osteoblastic MC3T3-E1 cells at subconfluency were cultured for 24 hr in a serum-free medium containing MK-7 (10^{-7}—10^{-5} M). Culture with MK-7 (10^{-6} or 10^{-5} M) caused a significant increase in protein content, alkaline phosphatase activity, and DNA content in osteoblastic cells.[69] The effect of MK-7 on cellular alkaline phosphatase activity and osteocalcin content was completely prevented with cycloheximide. MK-7 has a stimulatory effect on osteoblastic bone formation due to increasing protein synthesis.

Effects of MK-7 on Osteoclastic Bone Resorption Culture with bone-resorbing factors (PTH or PGE_2) caused a significant decrease in calcium content in the femoral-metaphyseal tissues obtained from young and aged rats *in vitro*.[70,71] The bone-resorbing factor-induced decrease in metaphyseal calcium content was completely prevented with MK-7 (10^{-7}—10^{-5} M) culture, indicating that MK-7 has an inhibitory effect on bone resorption in bone tissue culture. In addition, MK-7 (10^{-7}—10^{-5} M) completely prevented the PTH- or PGE_2-induced increase in medium glucose consumption and lactic acid production by bone tissues *in vitro*.[71] The preventive effect of MK-7 on bone resorption may partly result from the inhibition of the PTH- or PGE_2-stimulated medium glucose consumption and lactic acid production by bone tissues. PTH can stimulate extracellular release of lactic acid from osteoclastic cells. The PTH-induced increase in lactic acid production may result from the consumption of glucose by osteoclastic cells. This acid production is important as a cellular mechanism of bone resorption.

Osteoclast-like cells are formed from bone marrow cells in the presence of bone-resorbing factors (PTH or PGE_2). The PTH- or PGE_2-induced increase in osteoclast-like cell formation is significantly inhibited by MK-7 (10^{-8}—10^{-5} M).[70] MK-7 has a more potent inhibitory effect at the later stage of differentiation of marrow cells. Osteoclast-like cell formation was stimulated when dibutyryl cyclic AMP (DcAMP) or phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, was added to the culture. MK-7 significantly inhibited the effect of PMA on osteoclast-like cell formation, although it did not have an inhibitory effect on the DcAMP-induced increase in osteoclast-like cell formation. These observations suggest that the inhibitory action of MK-7 is partly involved in protein kinase C signaling.[70]

The effect of MK-7 on mature osteoclasts isolated from rat femoral tissues was examined.[70] Culture with MK-7 (10^{-6} or 10^{-5} M) caused a significant decrease in the number of mature osteoclasts. Such a decrease was also seen in the presence of calcitonin, DcAMP, or calcium chloride. The effect of MK-7 in decreasing the number of osteoclasts was not further enhanced in the presence of calcitonin, DcAMP, or calcium chloride, and it was completely abolished in the presence of dibucaine or staurosporine, which are inhibitors of Ca^{2+}-dependent protein kinases. The suppressive effect of MK-7 on osteoclasts may be partly mediated through the pathway of Ca^{2+}- and cyclic AMP-dependent signaling.

Preventive Effects of MK-7 on Bone Loss MK-7 has been demonstrated to have a stimulatory effect on osteoblastic bone formation and an inhibitory effect on bone resorption, thereby increasing bone mass, as shown in Fig. 4. The preventive effect of dietary MK-7 on bone loss was investigated using OVX rats.[72,73] OVX rats were given experimental diets containing menaquinone-4 (MK-4; 12 mg/100 g diet) or menaquinone-7 (MK-7; 18.1 mg/100 g diet) for 24 days: MK-4 and MK-7 were equal in molar concentrations. This feeding caused a remarkable increase in MK-4 and MK-7 concentrations in the serum and femur of OVX rats. OVX-induced decreases in the femoral dry weight and femoral calcium content were prevented by feeding with dietary MK-4 or MK-7. In separate experiments, OVX rats were given experimental diets containing fermented soybeans (*natto*; including MK-7 9.4 μg/100 g diet) with or without added MK-7 (37.6 μg/100 g diet) for 77 days. Feeding produced a significant elevation of MK-4 and MK-7 concentrations in the serum of OVX rats. The decreases in the femoral dry weight and femoral calcium content induced by OVX were significantly prevented by feeding with diets containing *natto* with MK-7 added (37.6 μg/100 g diet). Such an effect is not seen with diets containing natto without MK-7 or both MK-7 and isoflavone. The intake of

dietary MK-7 had a preventive effect on bone loss caused by OVX. This effect may be partly caused by MK-4, which is formed by degradation of MK-7.

The effect of the prolonged intake of dietary MK-7 on bone loss in OVX rats was also investigated. OVX rats were given experimental diets containing fermented soybeans (including MK-7 9.4 μg/100 g diet) with or without supplemental MK-7 (containing 14.1 or 18.8 μg of MK-7/100 g diet) for 150 days. Feeding produced a significant elevation of the MK-7 serum concentration of OVX rats. The serum γ-carboxylated osteocalcin concentration was significantly decreased by OVX. This decrease was significantly prevented by the feeding of *natto* diets with supplemental MK-7 (18.8 μg/100 g diets). OVX caused a significant decrease in femoral dry weight, femoral calcium content, and mineral density. These decreases were significantly prevented by feeding with diets containing *natto* with MK-7 (total, 18.8 μg/100 g diet). The prolonged intake of *natto* with MK-7 has a preventive effect on bone loss induced by OVX. Dietary MK-7 may be useful in the prevention of osteoporosis.

Effects of MK-7 in Normal Individuals The change in circulating MK-7 and γ-carboxylated osteocalcin (Gla osteocalcin) concentrations in normal individuals with the intake of fermented soybean (*natto*) was examined.[74,75] Forty-eight volunteers (45 men and 3 women) were divided into three groups of 16 volunteers each (15 men and 1 women), and each group was given sequentially fermented soybeans (50 g) containing three different amounts of MK-7 once a day for 14 days as follows: either regular *natto* with MK-7 865 μg/100 g of *natto*, reinforced *natto* containing MK-7 1295 μg/100 g, or MK-7 1730 μg/100 g. Serum MK-7 was not found in normal individuals who had not eaten *natto*. Serum MK-7 and γ-carboxylated osteocalcin concentrations were significantly raised 7, 10, and 14 days after the start of the intake of reinforced *natto* containing MK-7 1295 or 1730 μg/100 g. Serum γ-carboxylated osteocalcin levels were not significantly elevated with the intake of regular *natto*, although serum MK-7 levels were significantly raised. Moreover, serum γ-carboxylated osteocalcin concentration was significantly elevated 14 days after the intake of *natto* containing either 1295 or 1730 μg of MK-7/100 g diet, as compared with that after regular *natto* intake. The intake of dietary MK-7 in reinforced *natto* can stimulate γ-carboxylation of osteocalcin, which plays an important role in bone formation in normal individuals.

Epidemiologic data indicate that the intake of *natto* may play a role in the prevention of osteoporosis.[76] However, it has not been determined whether the prolonged intake of dietary *natto* causes an increase in MK-7 and a corresponding increase in γ-carboxylated osteocalcin in the serum of normal individuals. The appropriate amount of dietary MK-7 may have a stimulatory effect on bone mineralization in those individuals. The intake of reinforced *natto* that contains more MK-7 than regular *natto* may participate in the prevention of age-related bone loss.

A combination of nutritional factors may have an additive or synergistic effect on bone loss with increasing age. Zinc has been shown to enhance the effect of MK-7 in increasing bone calcium-content *in vitro*.[67] The combined administration of zinc and MK-7 was found to have a synergistic or additive enhancing effect on bone components in the femoral tissues of female elderly rats.[77] Supplemental intake of zinc and MK-7 may be useful in preventing osteoporosis with increasing age.

β-CRYPTOXANTHIN AND BONE METABOLISM

Retinol (vitamin A) is known to have a detrimental effect on bone at high doses. In laboratory animals, high levels of vitamin A lead to accelerated bone resorption, bone fractures, and osteoporotic bone lesions.[78—80] The effect of carotenoids on bone metabolism, however, have not been fully clarified. Carotenoids are present in fruit and vegetables.

Of the various carotinoids and rutin examined, β-cryptoxanthin was found to have a unique anabolic effect on bone calcification *in vitro*.[81] β-Cryptoxanthin is carotenoid abundant in Satsuma mandarin (*Citrus unshiu* MARC), and it is enzymatically converted from β-caroteine (provitamin A) in plants. The biological effect of β-cryptoxanthin has not been fully clarified. The chemical structure of β-cryptoxanthin is shown in Fig. 5.

Effects of β-Cryptoxanthin on Osteoblastic Bone Formation The effects of various carotenoids and rutin on calcium content and alkaline phosphatase activity in the femoral-diaphyseal (cortical bone) and metaphyseal (trabecular bone) tissues of young rats *in vitro* were investigated. Culture with β-cryptoxanthin (10^{-7} or 10^{-6} M) caused a significant increase in calcium content and alkaline phosphatase activity in

the femoral-diaphyseal and -metaphyseal tissues. Lutein, lycopene, and rutin 10^{-8} to 10^{-6} M did not have anabolic effects on calcium contents and alkaline phosphatase activity in rat femoral-diaphyseal and -metaphyseal tissues. β-Carotene (10^{-6} or 10^{-5} M) had no effect on the diaphyseal and metaphyseal calcium contents. β-Cryptoxanthin had a unique anabolic effect on bone calcification *in vitro*.[81] Moreover, culture with β-cryptoxanthin (10^{-6} or 10^{-5} M) caused a significant increase in DNA content in the femoral tissues.[82]

It has been reported that the serum concentration of β-cryptoxanthin due to consumption of vegetable juice in women is in the range of 1.3×10^{-7} to 5.3×10^{-7} M.[83] β-Cryptoxanthin in the range of 10^{-8} to 10^{-6} M caused a significant anabolic effect on biochemical components in rat femoral tissues *in vitro*, suggesting a physiologic role in the regulation of bone metabolism.

β-Cryptoxanthin was found to stimulate the proliferation of osteoblastic cells in subconfluent monolayers in a medium containing 10% FBS.[84] Culture with β-cryptoxanthin also caused a significant increase in biochemical components of osteoblastic cells.[84] The effect of β-cryptoxanthin on osteoblastic cell components seems to be partly mediated through protein kinase C or MAP kinase in the cells, since the carotenoid effects are inhibited by various inhibitors of intracellular signaling factors.

The effects of β-cryptoxanthin in increasing protein content, alkaline phosphatase activity, and DNA content in osteoblastic cells are completely inhibited in the presence of DRB, an inhibitor of RNA polymerase II. This suggests that β-cryptoxanthin has a stimulatory effect on transcriptional activity in osteoblastic cells. β-Cryptoxanthin is enzymatically converted from β-caroteine (provitamin A) in plants. Vitamin A (retinol) may be able to bind to nuclear receptors. It is speculated that β-cryptoxanthin binds to nuclear receptors in osteoblastic cells, and that the carotenoid transcriptional activity occurs in the cells.

Culture with β-cryptoxanthin stimulates the expression of insulin-like growth factor (IGF-I) or transforming growth factor (TGF)-β1 mRNA in osteoblastic cells using RT-PCR analysis.[84] This finding may support the view that β-cryptoxanthin has a stimulatory effect on transcriptional activity in osteoblastic cells. IGF-I or TGF-β1, which is a bone growth factor, is produced from osteoblasts.[85,86] The anabolic effect of β-cryptoxanthin in osteoblastic cells may be partly mediated through the action of IGF-I or TGF-β1 produced from the cells.

β-Cryptoxanthin (10^{-7} or 10^{-6} M) has also been found to increase the expression of Runx2, α1 (I) collagen, and alkaline phosphatase mRNAs in osteoblastic MC3T3-E1 cells.[87] Runx2 (Cbfa 1) is a member of the runt domain family of transcription factors, and it is involved in bone development.[88] α1 (I) collagen is a matrix protein that is related to bone formation and mineralization in osteoblast lineage cells.[89] Alkaline phosphatase participates in the mineralization process in osteoblastic cells.[90] β-Cryptoxanthin has a stimulatory effect on the expression of genes for proteins involved in osteoblasic bone formation.

The effects of β-cryptoxanthin in stimulating Runx2, α1 (I) collagen, and alkaline phosphatase mRNA expression in osteoblastic MC3T3-E1 cells was found to be prevented completely in the presence of DRB.[87] This result may support the view that β-cryptoxanthin stimulates transcriptional activity in osteoblastic MC3T3-E1 cells.

Vitamin A has been shown to stimulate the function of mouse osteoblastic cells.[91] Retinol and β-carotene inhibit the proliferation of osteoblastic MC3T3-E1 cells as well as DNA synthesis of the cells. Retinol induces differentiation of MC3T3-E1 cells by increasing alkaline phosphatase activity dose dependently (10^{-9}—10^{-7} M).[91] We confirmed that vitamin A (10^{-7} or 10^{-6} M) increases alkaline phosphatase activity in osteoblastic cells. β-Cryptoxanthin (10^{-7} or 10^{-6} M) caused a significant increase in alkaline phosphatase activity and protein content in osteoblastic cells. This effect of β-cryptoxanthin was also seen in the presence of vitamin A (10^{-6} M). Moreover, the stimulatory effect of β-cryptoxanthin on the expression of Runx2 type 1 and α1 (I) collagen mRNA was also observed in the presence of vitamin A. Vitamin A did not have a significant effect on Runx2 type 1 mRNA expression in osteoblastic MC3T3-E1 cells. Thus the mode of action of β-cryptoxanthin on gene expression in osteoblastic cells may differ from that of vitamin A, which is mediated through the RXR receptor in the nucleus of the cells.[92] It is speculated that β-cryptoxanthin may be able to bind other receptors (including orphan receptors), and that the carotenoid may stimulate transcriptional activity in osteoblastic cells.

Prolonged culture with β-cryptoxanthin is found to

stimulate mineralization. β-Cryptoxanthin significantly stimulated cell number, DNA content, protein content, and alkaline phosphatase activity in osteoblastic MC3T3-E1 cells when the cells were cultured for 3 to 21 days.[84] β-Cryptoxanthin has a stimulatory effect on cell differentiation and mineralization in osteoblastic cells.

The process of the action of β-cryptoxanthin in stimulating mineralization in osteoblastic cells is summarized in Fig. 6.

Effects of β-Cryptoxanthin on Osteoclastic Bone Resorption Culture with the bone-resorbing factor PTH or PGE_2 caused a significant decrease in calcium content in the diaphyseal and metaphyseal tissues. This decrease was completely inhibited by β-cryptoxanthin (10^{-8}—10^{-6} M). In addition, β-cryptoxanthin completely inhibited the PTH- or PGE_2-induced increase in medium glucose consumption and lactic acid production by bone tissues.[82] β-Cryptoxanthin has an inhibitory effect on bone resorption in tissue culture *in vitro*.

β-Cryptoxanthin (10^{-8}—10^{-6} M) has been shown to have a potent inhibitory effect on osteoclast-like cell formation in mouse marrow culture *in vitro*.[93] Culture with β-cryptoxanthin caused a marked inhibition of osteoblast-like cell formation induced in the presence of PTH, PGE_2, VD_3, LPS, or tumor necrosis factor-α (TNF-α). The inhibitory effect of β-cryptoxanthin was equal to that of 17β-estradiol, calcitonin, genistein, and zinc sulfate, which can inhibit osteoclast-like cell formation induced by bone-resorbing factors.

The inhibitory effect of β-cryptoxanthin on osteoclast-like cell formation was seen at the later stage of osteoclast differentiation in bone marrow cultures. This suggests that the carotenoid has a potent effect on the process of differentiation from mononuclear osteoclast to osteoclast. The receptor activator of NF-κB ligand (RANKL) acts on osteoclast progenitors, and the cytokine stimulates osteoclast differentiation.[94,95] RANKL plays a pivotal role in osteoclast differentiation. β-Cryptoxanthin had a significant inhibitory effect on osteoclast-like cell formation induced by RANKL.

RANKL expression is induced in osteoblastic cells and bone marrow stromal cells in response to osteoporotic factors, such as PTH, PGE_2, and VD_3, and combined treatment of hematopoietic cells with macrophage colony-stimulating factor (M-CSF) and the soluble form of RANKL (sRANKL) induces osteoclast differentiation *in vitro*. The receptor protein RANK is expressed on the surface of osteoclast progenitors. The supporting cells (osteoblasts or stromal cells) are not required for this activity. β-Cryptoxanthin significantly inhibited osteoclast-like cell formation induced by PTH, PGE_2, and VD_3 in mouse marrow cultures. Presumably, the inhibitory effect of the carotenoid is partly involved in RANKL expression, which is related to the effects of PTH, PGE_2, or VD_3.

LPS- or TNFα-induced osteoclast-like cell formation in mouse marrow cultures was significantly prevented by β-cryptoxanthin.[93] TNF-α is an autocrine factor in osteoclasts, promoting their differentiation and mediates RANKL induction of osteoclastogenesis.[96] TNF-α has also been shown to mediate *via* its p55 receptor in LPS-stimulated osteoclastogenesis. The inhibitory effect of β-cryptoxanthin on TNFα- or LPS-stimulated osteoclastogenesis may be a combination of effects by locally produced RANKL and LPS or TNF-α.

The interaction of RANKL with its receptor RANK leads to the recruitment of the signaling adaptor molecules TNF receptor-associated factors (TRAFs) to the receptor complex and the activation of NF-κB or c-Jun *N*-terminal kinase (JNK).[97] A protein kinase C family enzyme has a role in the regulation of osteoclast formation and function, potentially by participating in the extracellular signal-regulated kinase (ERK) signaling pathway of M-CSF and RANKL.

Phorbol 12-myristate 13-acetate (PMA) is an activator of protein kinase C. PMA significantly stimulated osteoclast-like cell formation in mouse marrow cultures, and the PMA-induced osteoclastogenesis was significantly inhibited by β-cryptoxanthin.[93] β-Cryptoxanthin may have a suppressive effect on the cellular response that is mediated through the binding of RANKL to RANK receptors in osteoclastogenesis.

In addition, β-cryptoxanthin did not have an inhibitory effect on RANKL expression in osteoblastic cells.[93] It is possible that β-cryptoxanthin has an effect on the expression of osteoprotegerin (ORG), a regulated suppressor of osteoclast differentiation, in osteoblasts. The action of β-cryptoxanthin in inhibiting osteoclastogenesis is summarized in Fig. 7.

Effects of β-Cryptoxanthin on Mature Osteoclasts The effects of β-cryptoxanthin on mature osteoclasts were investigated. M-CSF-dependent bone marrow macrophages were cultured in the presence of M-CSF

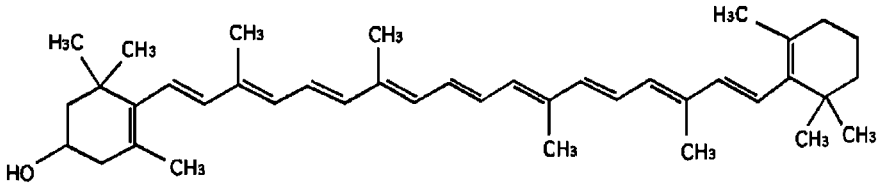

Fig. 5. Chemical Structure of β-Cryptoxanthin
The molecular weight of this compound ($C_{40}H_{56}O$) is 552.

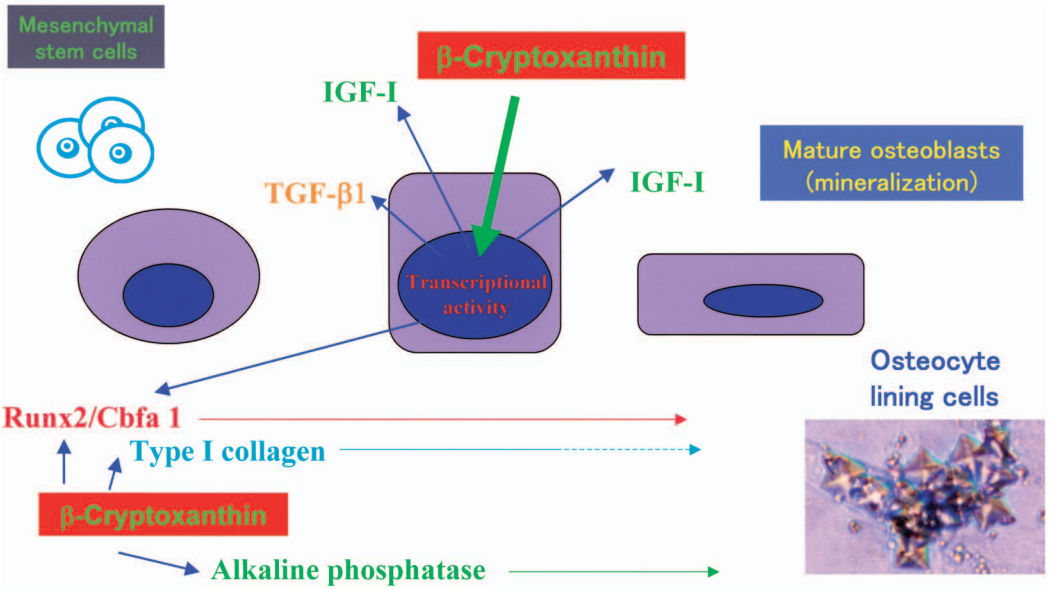

Fig. 6. Mechanism by which β–Cryptoxanthin Stimulates Cell Differentiation and Mineralization in Osteoblastic Cells
β–Cryptoxanthin stimulates the gene expressions of Runx2, a transcription factor related to cell differentiation, and α1 (I) collagen and alkaline phosphatase, which stimulate mineralization, in osteoblastic cells. It is speculated that β-cryptoxanthin binds to the orphan receptor in the nucleus.

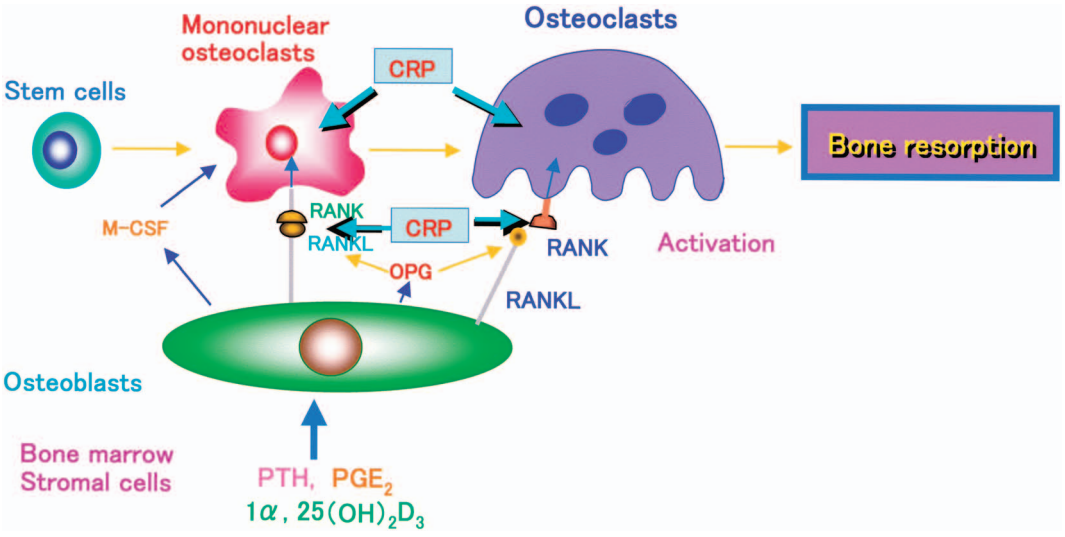

Fig. 7. Mechanism by which β-Cryptoxanthin Inhibits Osteoclastic Bone Resorption
β-Cryptoxanthin inhibits osteoclast-like cell formation in bone marrow culture systems, and stimulates apoptotic cell death and inhibits bone-resorbing activity in mature osteoclasts. β-Cryptoxanthin has an effect on their related gene expression. β-Cryptoxanthin may have an inhibitory effect on the signaling process of RANKL. Thus β-cryptoxanthin inhibits bone resorption.

and RANKL for 4 days.[98] The osteoclastic cells formed were further cultured in medium containing β-cryptoxanthin with or without M-CSF and RANKL for 24—72 h. Osteoclastic cells were significantly decreased in culture with β-cryptoxanthin (10^{-7} or 10^{-6} M) with or without M-CSF and RANKL for 72 h. The β-cryptoxanthin-induced decrease in osteoclastic cells was significantly inhibited in the presence of caspase-3 inhibitor. Agarose gel electrophoresis showed the presence of low-molecular-weight DNA fragments of adherent cells cultured with β-cryptoxanthin, indicating that the carotenoid induces apoptotic cell death.

Apoptosis-related gene expression was determined using RT-PCR.[98] Culture with β-cryptoxanthin caused a significant increase in caspase-3 mRNA expression in the presence or absence of M-CSF and RANKL, while Bcl-2 and Apaf-2 mRNA expressions were significantly increased in culture with β-cryptoxanthin without M-CSF and RANKL. Akt-1 mRNA expression was not significantly changed with culture when the carotenoid.

The expression of caspase-3 mRNA or Apaf-2, which involves apoptosis, in osteoclastic cells was found to be stimulated when cultured with β-cryptoxanthin in the absence of M-CSF and RANKL.[98] β-Cryptoxanthin stimulated caspase-3 mRNA expression in the presence of M-CSF and RANKL expression in the presence of M-CSF and RANKL.[98] β-Cryptoxanthin-induced apoptotic cell death is partly mediated through caspase-3 expression in osteoclastic cells. In addition, the expression of Bcl-2 mRNA, which is involved in rescue of apoptosis, is significantly decreased in β-cryptoxanthin culture in the presence or absence of M-CSF and RANKL.[98] However, Akt-1 mRNA expression is not significantly changed in culture with β-cryptoxanthin. The decrease in Bcl-2 mRNA expression may partly contribute to the effect of β-cryptoxanthin in stimulating the apoptotic cell death of osteoclastic cells.

Culture with β-cryptoxanthin was found to have suppressive effects on tartrate-resistant acid phosphatase (TRACP) activity, TRACP, and cathepsin K mRNA expression in osteoclastic cells in the presence or absence of M-CSF and RANKL. Presumably, β-cryptoxanthin has inhibitory effects on the activation of mature osteoclasts. β-Cryptoxanthin has stimulatory effects on apoptotic cell death and suppressive effects on osteoclastic cell function.

Preventive Effects of β-Cryptoxanthin against Bone Loss β-Cryptoxanthin has been shown to have a stimulatory effect on osteoblastic bone formation and an inhibitory effect on osteoclastic bone resorption *in vitro*.[82] The anabolic effect of β-cryptoxanthin on bone components *in vivo* was investigated. β-Cryptoxanthin (10, 25, or 50 μg/100 g body weight) was orally administered once daily for 7 days to young male rats.[99] The administration of β-cryptoxanthin (10, 25, or 50 μg/100 g body weight) caused a significant increase in calcium content and alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues. Femoral-diaphyseal and -metaphyseal DNA contents were significantly increased by the dose of 25 or 50 μg/100 g body weight. β-Cryptoxanthin has an anabolic effect on bone components in rats *in vivo*. Such an effect is also observed in the femoral tissues of aged (50-week-old) female rats.[100]

To determine whether β-cryptoxanthin has a preventive effect on bone loss in the pathophysiologic state, the effects of β-cryptoxanthin on bone components of streoptozotocin (STZ)-diabetic rats were determined.[101] Young rats received a single subcutaneous administration of STZ (6.0 mg/100 g body weight), and then the animals were orally administered β-cryptoxanthin (5 or 10 μg/100 g body weight) once daily for 7 or 14 days. The administration of STZ caused a significant decrease in body weight and a significant increase in serum glucose, triglyceride, and calcium levels, indicating a diabetic state. These alterations were significantly prevented by the administration of β-cryptoxanthin (5 or 10 μg/100 g) for 14 days. The administration of β-cryptoxanthin (5 or 10 μg/100 g) to normal rats for 14 days did not have a significant effect on body weight or on serum glucose, triglyceride, and calcium levels. Calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and -metaphyseal tissues were significantly decreased in STZ-diabetic rats. These decreases were significantly prevented by the administration of β-cryptoxanthin (5 or 10 μg/100 g) for 14 days. Thus the intake of β-cryptoxanthin was found to have a preventive effect on bone loss in STZ-diabetic rats.

The effect of β-cryptoxanthin on OVX-induced bone loss was examined.[102] β-Cryptoxanthin (5 or 10 μg/100 g body weight) was orally administered once daily for 3 months to OVX rats. The analysis using peripheral quantitative computed tomography

(pQCT) showed that OVX induced a significant decrease in mineral content and mineral density in the femoral-diaphyseal and -metaphyseal tissues. These decreases were significantly prevented by the administration of β-cryptoxanthin (5 or 10 μg/100 g). Moreover, OVX induced a significant decrease in calcium content and alkaline phosphase activity in the femoral-diaphyseal and -metaphyseal tissues and DNA content in the metaphyseal tissues. These decreases were significantly prevented by the administration of β-cryptoxanthin (5 or 10 μg/100 g). β-Cryptoxanthin has a preventive effect on OVX-induced bone loss *in vivo*.

Effects of β-Cryptoxanthin in Normal Individuals Changes in circulating biochemical markers of bone metabolism in normal individuals with the intake of juice prepared from Satsuma mandarin containing β-cryptoxanthin were investigated. Twenty-one volunteers (10 men and 11 women) were divided into two groups of 10 volunteers (5 men and 5 women) and 11 volunteers (5 men and 6 women), and each group was sequentially given juice (192 ml) containing two different amounts of β-cryptoxanthin once a day for 28 or 56 days: either regular juice with naturally occurring β-cryptoxanthin 802 μg/100 ml or a reinforced juice containing β-cryptoxanthin 1500 μg/100 ml.[103] As serum bone markers, bone-specific alkaline phosphatase, γ-carboxylated osteocalcin, bone TRACP, and *N*-telopeptide of type I collagen were determined. The intake of regular juice for 28 or 56 days caused a significant increase in γ-carboxylated osteocalcin, a marker of bone formation. Intake for 56 days produced a significant decrease in serum bone TRACP activity. Moreover, the intake of the β-cryptoxanthin-reinforced juice for 28 or 56 days caused a significant increase in the serum γ-carboxylated osteocalcin concentration and a corresponding decrease in serum bone TRACP activity and *N*-telopeptide of type I collagen, a marker of bone resorption. The intake of β-cryptoxanthin-reinforced juice thus has a stimulatory effect on bone formation and an inhibitory effect on bone resorption in normal individuals.[103]

The serum β-cryptoxanthin concentration was significantly increased with the intake of regular juice for 56 days. This increase was significantly enhanced by the intake of β-cryptoxanthin-reinforced juice.[104] A positive relationship between serum β-cryptoxanthin and circulating γ-carboxylated osteocalcin concentration was found using the value obtained from all groups for before intake and with the intake of regular juice and β-cryptoxanthin-reinforced juice. A negative relationship between serum β-cryptoxanthin concentration and circulating TRACP activity was observed. A relationship between serum β-cryptoxanthin and circulating bone metabolic markers was found in healthy individuals with the intake of juice containing β-cryptoxanthin.[104]

Thus the intake of dietary β-cryptoxanthin may have a preventive effect on bone loss in humans with increasing age.

EFFECTS OF OTHER FOOD AND PLANT FACTORS ON BONE METABOLISM

As mentioned above, isoflavones, menaquinone-7, and β-cryptoxanthin have been shown to have a stimulatory effect on bone formation and an inhibitory effect on bone resorption, thereby increasing bone mass. These food factors may have a preventive effect on bone loss with increasing age. Moreover, the identification of other food and plant factors may be important in the prevention of osteoporosis. Further study was undertaken to determine the effect of various food and plant extracts on bone metabolism.

Effects of Wasabi Leafstalk Extract on Bone Metabolism The effects of 20% ethanol extracts obtained from various food and plants on bone calcium content were examined using bone tissues *in vitro*. Of various food and plants (including *katsuo-bushi*, loquat leaf, cherry leaf, dried shiitake, raw shiitake, gabaron tea, green tea, muskmelon, tomato, and blueberry) examined, wasabi leafstalk (*Wasabi japonica* MATSUM) extract was found to have an anabolic effect on bone calcification in mouse calvaria tissue culture *in vitro*.[105] Wasabi leafstalk extract was obtained from a homogenate with 20% ethanol. The presence of wasabi leafstalk extract (10 μg/ml) caused a significant increase in calcium content and alkaline phosphatase activity in bone tissues. With higher concentrations (50 and 250 μg/ml), however, the effect was weakened. The wasabi leafstalk extract-induced increase in bone calcium content was completely prevented by coculture with cycloheximide, an inhibitor of protein synthesis, suggesting that the effect of wasabi leafstalk extracts is based on newly synthesized protein components. In addition, wasabi leafstalk has an inhibitory effect on PTH-induced osteoclast-like cell formation in a mouse marrow culture system, suggesting that the component inhibits

bone resorption (unpublished data).

The stimulatory effect of wasabi leafstalk extract on bone calcification was compared with the anabolic effects of insulin, IGF-I, 17β-estradiol, and genistein.[106] The combination of 10^{-10} M of 17β-estradiol and 15 μg/ml of wasabi leafstalk extract had an additive effect on bone calcium content. The combination of either insulin 10^{-8} or 10^{-7} M with wasabi leafstalk extract (15 μg/ml) had an additive effect. Such an effect was not seen in the case of IGF-I (10^{-8} M). Wasabi leafstalk extract has an enhancing effect on the anabolic action of 17β-estradiol or insulin, which physiologically regulates bone formation and calcification *in vitro*.

The components of the wasabi leafstalk extracts which increase bone calcium content were stable when treated with heating or with acidity or alkalinization.[107] The active component of wasabi leafstalk extract thus may not be a protein.

The active component in wasabi leafstalk was purified.[108] Wasabi leafstalk extract was obtained from a homogenate with 20% ethanol. The active component, which was found in the ethanol extract, was purified upon gel filtration chromatography with a HiLoad 26/80 Superdex 30-pg column and reverse-phase chromatography on a Resource 15 RPC 3-ml column. The result of ESI mass spectra of the purified active component showed that its material had a molecular weight of 158. The material with a low molecular weight of 158 was the active component in wasabi leafstalk which stimulates bone calcification. The identification of chemical structure is in progress.

To determine the anabolic effect of wasabi leafstalk extract on bone components *in vivo*, extracts (10—40 mg/100 g body weight) were orally administered once daily for 7 days in young and aged rats.[109,110] The biochemical components in the femoral-diaphyseal and -metaphyseal tissues of young and aged rats was significantly increased by the administration of wasabi leafstalk extract for 7 days *in vivo*. Meanwhile, body weight, serum calcium, and inorganic phosphorus concentrations of the rats were not significantly altered by the administration of wasabi leafstalk extract for 7 days. Wasabi leafstalk extract therefore has an anabolic effect on bone components *in vivo*. The intake of wasabi leafstalk extract may have a preventive effect on bone loss.

Effects of Marine Alga Extract on Bone Metabolism The effect of marine algae on bone metabolism has not yet been clarified. We determined the effects of various marine algae on bone calcification in the femoral tissues of rats *in vitro*.[111] *Undaria pinnatifida*, *Sargassum horneri*, *Eisenia bicyclis*, *Cryptonemia scmitziana*, *Gelidium amansii*, and *Ulva pertusa* Kjellman, which were gathered seasonally, were used. Water suspensions (5%) of marine alga powder were orally administered once daily for 7 days. Bone calcium content was significantly increased by the administration of *U. pinnatifida*, *S. horneri*, *E. bicyclis*, or *C. scmitziana*. Bone alkaline phosphatase activity, which is an enzyme for calcification, was significantly enhanced with the administration of *S. horneri* or *G. amansii*. Femoral-metaphyseal tissues were cultured in medium containing water-solubilized extract (25 and 50 μg/ml) obtained from *U. pinnatifida*, *S. horneri*, *E. bicyclis*, and *C. scmitziana*. The bone calcium content was significantly elevated in the presence of *S. horneri* extract (25 and 50 μg/ml). No effect was seen with the other extracts. *S. horneri* extract has a unique anabolic effect on bone calcification *in vivo* and *in vitro*. The effects of *S. horneri* extract in increasing calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and -metaphyseal tissues was completely abolished in the presence of cycloheximide, an inhibitor of protein sythesis.[112] The anabolic effect of *S. horneri* extract may be based on newly synthesized protein components.

The effect of *S. horneri* extract on bone resorption *in vitro* was examined.[113] The PTH- and PGE_2-induced decrease in bone calcium content was completely inhibited by water-solubilized extracts (10, 25, and 50 μg/ml) of *S. horneri*. In addition, these extracts (25 and 50 μg/ml) completely inhibited the PTH- or PGE_2-induced increase in medium glucose consumption and lactic acid production by bone tissues. Moreover, *S. horneri* extracts (10—50 μg/ml) blocked the PTH-induced increase in acid phosphatase activity in the diaphyseal and metaphyseal tissues. These findings indicate that water-solubilized extracts of *S. horneri* have a direct inhibitory effect on bone resorption in tissue culture *in vitro*.

S. horneri extract has been found to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption *in vitro*.[111—113] Those active components are found to be present in *S. horneri* obtained from various coasts in Japan (Shimoda and Iwate) and China.[114]

The active component of *S. horneri* extract in stimulating bone calcification is found to be near MW 1000. The effect of this component disappeared with heat treatment of the extract. Meanwhile, the active component of *S. horneri* extract in inhibiting osteoclastic cell formation was more than MW 50000. This component was stable under heat treatment. It is speculated that the active component in stimulating bone calcification is a peptide or chemical, and that the compound in inhibiting bone resorption is a polysaccharide. These active components obtained from the coasts of Iwate or China showed an identical molecular weight. Thus the active components are found in *S. horneri* extracts obtained from different coasts. Interestingly, the two active components are present in *S. horneri* extract.

The anabolic effects of *S. horneri* extract on bone components in the femoral tissues of young and aged rats *in vivo* was demonstrated.[115] Calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and -metaphyseal tissues of young male (4-week-old) rats was significantly increased with the administration of *S. horneri* extract (2.5, 5, and 10 mg/100 g) for 7 days. Moreover, these bone components in the femoral-diaphyseal and -metaphyseal tissues of aged female (50-week-old) rats were significantly increased by the administration of *S. horneri* extract (10 mg/100 g) for 14 days, suggesting a preventive effect on bone loss with increasing age.

Wheter the intake of *S. horneri* extract has a preventive effect on bone loss in a pathophysiologic state was examined.[116] Diabetes has been shown to induce bone loss.[117,118] The oral administration of *S. horneri* extract to STZ-diabetic rats was found to have a preventive effect on bone loss with diabetes *in vivo*.[116] This finding suggests that the dietary intake of *S. horneri* extract has a preventive effect on bone loss in the pathophysiologic state. When the femoral tissues obtained from STZ-diabetic rats were cultured in medium containing *S. horneri* extract solution, the femoral calcium content and alkaline phosphatase activity were significantly increased *in vitro*. Alkaline phosphatase is related to bone calcification. *S. horneri* extract has been shown to stimulate bone formation and to inhibit bone resorption *in vitro*. Presumably, the preventive effect of *S. horneri* extract administration on diabetes-induced bone loss is related to a direct action of the active component of *S. horneri* extract.

Interestingly, the oral administration of *S. horneri* extract to STZ-diabetic rats was found to have a significant preventive effect on the decrease in body weight and the increase in serum glucose and triglyceride levels induced in the diabetic state.[114] This is a novel finding. The intake of *S. horneri* extract has a partial restorative effect on serum biochemical finding with diabetes *in vivo*. Whether the active component of *S. horneri* extract in preventing bone loss induced with diabetes is identical to the component that prevents an elevation of serum glucose and triglyceride levels with diabetes is unknown. Thus the intake of *S. horneri* extract has been demonstrated to have preventive effects on bone loss, hyperglycemia, and hyperlipidemia in STZ-diabetic rats.

Effects of Bee Pollen Extract on Bone Metabolism Bee pollen is generally used as a nutrient factor. The role of bee pollen in the prevention of bone loss with increasing age has not yet been clarified. Bee pollen extract was found to have anabolic effects on bone components in the femoral-diaphyseal and -metaphyseal tissues of rats *in vitro* and *in vivo*.[119] Of various bee pollens, the water-solubilized extract obtained from *Cistus ladaniferus* had a marked effect in increasing the bone calcium content. The ethanol-solubilized extract of bee pollen also significantly increased the bone calcium content. However, the effects of the water-solubilized extract in increasing the bone calcium content were greater than those of the ethanol-solubilized extract. The active component in bee pollen was thus present in water-solubilized extract. Culture with the extract obtained from the pollen of *C. ladaniferus* caused a significant increase in alkaline phosphatase activity and DNA content in the femoral-diaphyseal and -metaphyseal tissues *in vitro*. These increases were completely inhibited in the presence of cycloheximide, suggesting that its stimulatory effect results from newly synthesized protein components.

The water-solubilized extract of bee pollen obtained from *C. ladaniferus* has been shown to have inhibitory effects on bone resorption in femoral tissues and osteoclast-like cell formation in bone marrow cell culture *in vitro*.[120] Bee pollen extract has stimulatory effects on bone formation and inhibitory effects on bone resorption *in vitro*.

The effects of bee pollen, propolis, and royal jelly extracts were compared. The effects of water-solubi-

lized extract or ethanol-solubilized extract obtained from propolis on bone calcium content *in vitro* were prepared.[119] The water-solubilized extract (100 μg/ml of medium) caused a significant increase in bone calcium content. The ethanol-solubilized extract (10, 50, or 100 μg/ml) did not have significant effects on bone calcium content. Meanwhile, royal jelly (50 or 100 μg/ml) significantly increased calcium content in rat femoral tissues *in vitro*.[121] However, culture with royal jelly (1—100 μg/ml) did not have a suppressive effect on PTH-induced increase in osteoclast-like cell formation in bone marrow culture *in vitro*.[121] Among bee-related factors, bee pollen extract has a potent anabolic effect on bone calcification. The identification of the active component in bee pollen extracts is in progress.

The oral administration of the water-solubilized extract obtained from the pollen of *C. ladaniferus* to rats caused a significant increase in calcium content in the femoral-diaphyseal and -metaphyseal tissues, indicating that the extract exerts anabolic effects on bone components *in vivo*.[119] It is speculated that the active component in the water-solubilized extract obtained from the pollen of *C. ladaniferus* is transported in the intestine, and acts on osteoblastic cells in bone tissues. If the active component is transported in the intestine, it may not be a high molecular-weight compound. The active component in bee pollen extract is found in the fraction of less than MW 1000.[122]

Effects of Phytocomponent *p*-Hydroxycinnamic Acid on Bone Metabolism Cinnamic acid is present in many plant and fruits. *p*-Hydroxycinnamic acid is an intermediate-metabolic substance in plant and fruits and it is synthesized from tyrosine. The effect of cinnamic acid or its related compounds on bone metabolism has not yet been clarified.

Rat femoral-diaphyseal or -metaphyseal tissues were cultured for 48 h in medium containing cinnamic acid, *p*-hydroxycinnamic acid (HCA) ferulic acid, caffeic acid or 3,4-dimethoxycinnamic acid (DCA) *in vitro*.[123] Culture with HCA (10^{-5} or 10^{-4} M) caused a significant increase in calcium content in the diaphyseal and metaphyseal tissues. Such an effect was not observed in the presence of cinnamic acid or other compounds at the concentration of 10^{-5} or 10^{-4} M. Alkaline phosphatase activity and DNA content in the diaphyseal or metaphyseal tissues was significantly increased in the presence of HCA (10^{-5} or 10^{-4} M). The effects of HCA (10^{-4} M) in increasing calcium content, alkaline phosphatase activity, and DNA content in the diaphyseal or metaphyseal tissues were completely prevented in the presence of cycloheximide (10^{-6} M), an inhibitor of protein synthesis. HCA was found to have anabolic effects on bone components.

Culture with PTH caused a significant decrease in calcium content and a corresponding elevation in medium glucose consumption, lactic acid production, or TRACP activity in the diaphyseal or metaphyseal tissues. These alterations were completely prevented in the presence of HCA (10^{-5} or 10^{-4} M). Thus HCA was found to have inhibitory effects on bone resorption in tissue culture *in vitro*.[123]

The anabolic effects of phytocomponent HCA on the bone of rats *in vivo* were determined.[124] Rats were orally administered HCA 1, 2, or 5 mg/100 g body weight once daily for 7 days. The administration of HCA did not cause a significant change in body weight or serum calcium and inorganic phosphorus levels. Calcium content, alkaline phosphatase activity, and DNA content in the diaphyseal and metaphyseal tissues were significantly increased with the administration of HCA 2 or 5 mg/100 g. Diaphyseal calcium and metaphyseal DNA contents were significantly increased with the dose of HCA 1 mg/100 g. The activity of TRACP, which is a marker enzyme of osteoclastic bone resorption, was significantly decreased with the administration of HCA 1, 2, or 5 mg/100 g. The administration of HCA was found to have anabolic effects on bone calcification in the femoral tissues of rats *in vivo*.

Phycomponent HCA may have a preventive effect on bone loss with increasing age. Further study is in progress to determine a role in therapy of osteoporosis.

PROSPECTS

Bone loss with aging induces osteoporosis, and a decrease in bone mass leads to bone fracture. Bone loss may be due to decreased bone formation and increased bone resorption. Pharmacologic and food factors are needed to prevent bone loss with increasing age. Chemical factors in food and plants may help to prevent bone loss with aging.

Recent studies in our laboratory have shown that isoflavones (including genistein and daidzein), which are contained in soybeans, have a stimulatory effect

on osteoblastic bone formation and an inhibitory effect on osteoclastic bone resorption, thereby increasing bone mass. Also, menaquinone-7, an analogue of vitamin K_2 abundant in fermented soybeans, has been demonstrated to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption. Of various carotenoids (including lutein, lycopone, and β-carotene), β-cryptoxanthin, which is abundant in Satsuma mandarin (*C. unchiu* MARC), has a stimulatory effect on osteoblastic bone formation and an inhibitory effect on osteoclastic bone resorption. The supplementation of isoflavone, menaquinone-7, and β-cryptoxanthin has a preventive effect on bone loss induced by OVX in rats, which is an animal model of osteoporosis. These factors have been demonstrated to have anabolic effects on bone metabolism in humans. Food chemical factors play a role in bone health and may be important in the prevention of bone loss with increasing age.

Among other food and plants, factors that have an anabolic effect on bone metabolism are found in extracts obtained from wasabi leafstalk (*W. japonica* MATSUM), marine alga *S. horneri*, and bee pollen from *C. ladaniferus*. Phytocomponent HCA, which is present in many plants and fruit, has also been shown to have an anabolic effect on bone metabolism.

Drugs used clinically in the treatment and therapy of osteoporosis are mainly based on the action of osteoclastic bone resorption. A clinical compound that stimulates bone formation is under development. As described in this review, chemical factors that can stimulate bone formation are found in various food and plants. These factors may be usefulness in the prevention and therapy of osteoporosis. It is expected that studies of the chemical structure-related activity of biofactors from food and plants will lead to the development of new drugs that stimulate bone formation and inhibit bone resorption.

The supplemental intake of food and plant factors that increase bone mass may play a role in maintaining bone health and in prevention of bone loss with increasing age. Further studies are needed to identify food and plant factors that have an anabolic effect on bone metabolism.

REFERENCES

1) Nishimoto S. K., Chang C.-H., Gendler E., Stryker W. F., Nimni M. E., *Calcif. Tissue Int.*, **37**, 617–624 (1985).
2) Schapira C., Slinn S., Sarid M., Mokadai S., Kabala A., Silbermann M., *Bone*, **16**, 575–582 (1995).
3) Wild R. A., Buchamain J. R., Meyers C., Demers L. M., *Proc. Soc. Exp. Biol. Med.*, **186**, 335–360 (1987).
4) Cooper C., Melton III J., *Trends Endocrinol. Metab.*, **3**, 224–229 (1992).
5) Cummings S. R., Kelsey J. L., Nevitt M. C., O'Dowd K. J., *Epidemiol. Rev.*, **7**, 178–199 (1985).
6) Young G. A., Chem C., Hill G. L., *Am. J. Clin. Nutr.*, **31**, 429–435 (1978).
7) Older M. W. J., Delyth E., Dickerson J. W. T., *Br. J. Surg.*, **67**, 884–886 (1980).
8) Bonjour J.-P., Schurch M.-A., Rizzori R., *Bone*, **18**, 1395–1445 (1996).
9) Yamaguchi M., Gao Y. H., *Res. Exp. Med.*, **197**, 101–107 (1997).
10) Yamaguchi M., Gao Y. H., *Mol. Cell. Biochem.*, **178**, 377–382 (1998).
11) Erikson E. F., Colvard D. S., Berg N. J., Graham M. L., Mann K. G., Spelsberg T. C., Riggs B. L., *Science*, **241**, 84–86 (1988).
12) Komm B. S., Terpening C. M., Beng D. J., Graema K. A., Gallegos A., Korc M., Greene G. C., O'Malley B. W., Haussler M. R., *Science*, **241**, 81–83 (1988).
13) Gao Y. H., Yamaguchi M., *Mol. Cell. Biochem.*, **194**, 93–98 (1999).
14) Yamaguchi M., Ma Z. J., Fushimi T., *J. Bone Miner. Metab.*, **20**, 148–155 (2002).
15) Yamaguchi M., Ma Z. J., *Biol. Pharm. Bull.*, **24**, 1437–1439 (2001).
16) Sugimoto E., Yamaguchi M., *Int. J. Mol. Med.*, **5**, 515–520 (2000).
17) Sugimoto E., Yamaguchi M., *Biochem. Pharmacol.*, **59**, 471–475 (2000).
18) Yamaguchi M., Sugimoto E., *Mol. Cell. Biochem.*, **214**, 97–102 (2000).
19) Kuiper G. G. J. M., Carlsson B., Grandien K., Enmark E., Haggblad J., Nilsson S., Gustafsson J.-A., *Endocrinology*, **138**, 863–870 (1997).
20) Hoskinson R. M., Khorana H. G., *J. Biol. Chem.*, **240**, 2129–2134 (1965).
21) Miksicek R. J., *Mol. Pharmacol.*, **44**, 37–43 (1993).
22) Miksicek R. J., *J. Steroid Biochem. Mol.*

Biol., **49**, 153–160 (1994).
23) Klein-Nulend J., Fall P. M., Raisz L. G., *Endocrinology*, **126**, 223–227 (1990).
24) Klein D. C., Raisz L. G., *Endocrinology*, **86**, 1436–1440 (1970).
25) Meryon S. D., Perris A. D., *Life Sci.*, **28**, 1061–1065 (1981).
26) Klaushofer K., Hoffmann O., Stewart P. J., Caerwenka E., Koller K., Poterlik M., Stern P. H., *J. Pharmacol. Exp. Ther.*, **243**, 584–590 (1987).
27) Yamaguchi M, Gao Y. H., *Biochem. Pharmacol.*, **55**, 71–76 (1998).
28) Takahashi N., Yamana H., Yoshiki S., Roodman G. D., Mundy G. R., Jones S. J., Boyde A., Suda T., *Endocrinology*, **122**, 1373–1382 (1988).
29) Burstone M. S., *J. Natl. Cancer Inst.*, **21**, 523–539 (1958).
30) Minkin C., *Calcif. Tissue Int.*, **34**, 285–290 (1982).
31) Gao Y. H., Yamaguchi M., *Biochem. Pharmacol.*, **58**, 767–772 (1999).
32) Spinozzi F., Pagliacci M. C., Migliorati G., Miraca R., Grignami F., Roccardi C., Nicholetti I., *Leuk. Res.*, **18**, 431–439 (1994).
33) Gao Y. H., Yamaguchi M., *Biol. Pharm. Bull.*, **22**, 805–809 (1999).
34) Nicholson G. C., Linsey S. A., Moseley J. M., Martin T. J., *J. Clin. Invest.*, **78**, 355–360 (1986).
35) Su Y., Charkraborty M., Nathanson M. H., Baron R., *Endocrinology*, **131**, 1497–1502 (1992).
36) Jones D. P., McConkey D. J., Nicotera P., Orrenius S., *J. Biol. Chem.*, **264**, 6398–6403 (1989).
37) McCabe M. J., Orrenius S., *Biochem. Biophys, Res. Commum.*, **194**, 944–950 (1993).
38) Gao Y. H., Yamaguchi M., *Int J. Mol. Med.*, **5**, 261–267 (2000).
39) Liu Y., Bhalla K., Hill C., Priest D. G., *Biochem. Pharmacol.*, **48**, 1265–1272 (1994).
40) Aoki K., Didomenico E., Sims N. A., Mukhopadhyay K., Neff L., Houghton A., Amling M., Levy J. B., Horne W. C., Baron R., *Bone*, **25**, 261–267 (1999).
41) Omo R., Yamaguchi M., *J. Health Sci.*, **45**, 66–69 (1999).
42) Omo R., Yamaguchi M., *J. Health Sci.*, **45**, 251–255 (1999).
43) Riggs B. L., Jewsey J., Kelly P. J., Jones J. D., Maker F. T., *J. Clin. Invest.*, **48**, 1065–1072 (1969).
44) Kalu D. N., Liu C.-C., Hardin R. R., Hillis B. W., *Endocrinology*, **124**, 7–16 (1989).
45) Ono R., Ma Z. J., Yamaguchi M., *J. Health Sci.*, **46**, 70–74 (2000).
46) Yamaguchi M., Ono R., Ma Z. J., *J. Health Sci.*, **47**, 579–582 (2001).
47) Gao Y. H., Yamaguchi M., *Gen. Pharmacol.*, **31**, 199–202 (1998).
48) Yamaguchi M., Gao Y. H., Ma Z. J., *J. Bone Miner. Metab.*, **18**, 77–83 (2000).
49) Ma Z. J., Igarashi A., Inagaki M., Mitsugi F., Yamaguchi M., *J. Health Sci.*, **46**, 363–369 (2000).
50) Yamaguchi M., *Curr. Med. Chem.*, **1**, 356–365 (1995).
51) Yamaguchi M., *Gen. Pharmacol.*, **26**, 1179–1183 (1995).
52) Yamaguchi M., *J. Trace Elem. Exp. Med.*, **11**, 119–135 (1998).
53) Ma Z. J., Shimanuki S., Igarashi A., Kawasaki Y., Yamaguchi M., *J. Health Sci.*, **46**, 263–268 (2000).
54) Yamaguchi M., Igarashi A., Sakai M., Degaw. H., Ozawa Y., *J. Health Sci.*, **51**, 191–196 (2005).
55) Nagasawa T., Yuan Y. V., Kitts D. D., *Nutr. Res.*, **11**, 819–830 (1991).
56) Kitts D. D., Yuan Y. V., Nagasawa T., Moriyama Y., *Br. J. Nutr.*, **68**, 765–781 (1992).
57) Yamaguchi M., Tezuka M., Shimanuki S., Kishi M., Takada Y., *Food Sci. Technol. Int.*, **4**, 209–212 (1998).
58) Ma Z. J., Yamaguchi M., *J. Health Sci.*, **46**, 474–479 (2000).
59) Hauschka P. V., Lian J. B., Gallop P. M., *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 3925–3929 (1975).
60) Price P. A., *Vitam. Horm.*, **42**, 65–108 (1985).
61) Hauschka P. V., Carr S. A., *Biochemistry*, **21**, 2538–2547 (1982).
62) Hart J. P., Sheaver M. J., Klenerma L., Cat-

teral A., Reeve J., Sambrook P. N., Dodds R. A., Biotensky L., Chayen J., *J. Clin. Endocrinol. Metab.*, **60**, 1268–1269 (1985).
63) Knapen M. H. J., Hamulya K., Vermer C., *Ann. Intern Med.*, **111**, 1001–1005 (1989).
64) Koshihara Y., Hoshi K., Shivaki M., *J. Clin. Exp. Med.*, **161**, 439–440 (1992).
65) Hara K., Akiyama Y., Nakamura T., Murota S., Morita, I., *Bone*, **16**, 179–184 (1995).
66) Akiyama Y., Hara K., Ohkawa I., Tajima, T., *Jpn. J. Pharmacol.*, **62**, 145–153 (1993).
67) Ehara Y., Tahakashi H., Hanahisa Y., Yamaguchi M., *Res. Exp. Med.*, **196**, 171–178 (1996).
68) Yamaguchi M., Uchiyama S., Tsukamoto Y., *Int. J. Mol. Med.*, **10**, 729–733 (2002).
69) Yamaguchi M., Sugimoto E., Hachiya S., *Mol. Cell. Biochem.*, **223**, 131–137 (2001).
70) Yamaguchi M., Ma Z. J., *Mol. Cell. Biochem.*, **228**, 39–47 (2001).
71) Yamaguchi M., Uchiyama S., Tsukamoto Y., *Mol. Cell. Biochem.*, **245**, 115–120 (2003).
72) Yamaguchi M., Taguchi H., Gao Y. H., Igarashi A., Tsukamoto Y., *J. Bone Miner. Metab.*, **17**, 23–29 (1999).
73) Yamaguchi M., Kakuda H., Gao Y. H., Tsukamoto Y., *J. Bone Miner. Metab.*, **18**, 71–76 (2000).
74) Tsukamoto Y., Ichise H., Kakuda H., Yamaguchi M., *J. Bone Miner. Metab.*, **18**, 216–222 (2000).
75) Tsukamoto Y., Ichise H., Yamaguchi M., *J. Health Sci.*, **46**, 317–321 (2000).
76) Hosoi T., *Nippon Ronnen Igakkai Zasshi*, **33**, 240–244 (1996).
77) Ma Z. J., Igarashi A., Yamakawa K., Yamaguchi M., *J. Health Sci.*, **47**, 40–45 (2001).
78) Forsyth K. S., Watson R. R., Gensler H. L., *Life Sci.*, **45**, 2149–2156 (1989).
79) Rohde C. M., Monatt M., Clagett-Dame M., Deluca H. F., *J. Nutr.*, **129**, 2241–2250 (1999).
80) Promislow J. H. E., Goodman-Gruen D. Slymen D. J., Barret-Connor E., *J. Bone Miner. Res.*, **17**, 1349–1358 (2002).
81) Yamaguchi M., Uchiyama S., *Biol. Pharm. Bull.*, **26**, 1188–1191 (2003).
82) Yamaguchi M., Uchiyama S., *Mol. Cell. Biochem.*, **258**, 137–144 (2004).
83) McEligot A. J., Rock C. L., Shanks T. G., Flatt S. W., Newman V., Farber S., Pierce J. P., *Cancer Epideminol. Biomarkers Prev.*, **8**, 227–231 (1999).
84) Uchiyama S., Yamaguchi M., *Int. J. Mol. Med.*, **15**, 675–681 (2005).
85) Centrella M., McCarthy T. L., Canalis E., *Endocrinology*, **126**, 39–44 (1990).
86) Palcy S., Bolivar I., Goltzman D., *J. Bone Miner. Res.*, **15**, 2352–2361 (2000).
87) Uchiyama S., Yamaguchi M., *J. Cell. Biochem.*, **95**, 1224–1234 (2005).
88) Komori T., Yagi H., Nomura S., Yamaguchi A., Sasaki K., Deguchi K., Shimizu Y., Bronson R. T., Gao Y. H., Inada M., Sato M., Okamoto R., Kitamura Y., Yoshiki S., Kishimoto T., *Cell*, **89**, 755–764 (1997).
89) Lian J. B., Stein G. S., Canalis E., Roby P. G., Boskey A. L., ''Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism,'' ed. by Farus M. J., Lippincott Williams & Wilkins Press, New York, 1999, pp. 14–29.
90) Yokay D. A., Zhang J., Thrailkill K. M., Arthur J. M., Quarles L. D., *J. Cell. Physiol.*, **158**, 467–475 (1994).
91) Park C. K., Ishimi Y., Ohmura M., Yamaguchi M., Ikegami S., *J. Nutr. Sci. Vitaminol.* (Tokyo), **43**, 281–296 (1997).
92) MacDonald P. N., Dowd D. R., Nakajima S., Galligan M. A., Reeder M. C., Haussler C. A., Ozato K., Haussler M. R., *Mol. Cell. Biol.*, **13**, 5907–5917 (1993).
93) Uchiyama S., Yamaguchi M., *Biochem. Pharmacol.*, **67**, 1297–1305 (2004).
94) Zaidi M., Blair H. C., Moonga B. S., Abe E., Huang, C. L.-H., *J. Bone Miner. Res.*, **18**, 599–609 (2003).
95) Tanaka S., Nakamura I., Inoue J.-I., Oda H., Nakamura K., *J. Bone Miner. Metab.*, **21**, 123–133 (2003).
96) Zou W., Hakin I. Tschoep K., Endres S., Bar-Shavit Z., *J. Cell. Biochem.*, **83**, 70–83 (2001).
97) Kim H.-H., Kim H.-M., Kwack K., Kim S. W., Lee Z. H., *J. Biochem. Mol. Biol.*, **34**, 421–427 (2001).
98) Uchiyama S., Yamaguchi M., *J. Cell.*

Biochem., **98**, 1185–1195 (2006).
99) Uchiyama S., Sumida T., Yamaguchi M., *Biol. Pharm. Bull.*, **27**, 232–235 (2004).
100) Uchiyama S., Sumida T., Yamaguchi M., *J Health Sci.*, **50**, 491–496 (2004).
101) Uchiyama S., Yamaguchi M., *Biol. Pharm. Bull.*, **28**, 1766–1769 (2005).
102) Uchiyama S., Yamaguchi M., *Int. J. Mol. Med.*, **17**, 15–20 (2006).
103) Yamaguchi M., Igarashi A., Uchiyama S., Morita S., Sugawara K., Sumida K., *J. Health Sci.*, **50**, 619–624 (2004).
104) Yamaguchi M., Igarashi A., Morita S., Sumida T., Sugawara K., *J. Health Sci.*, **51**, 738–743 (2005).
105) Suzuki T., Nakayama H., Yamaguchi M., *Food Sci. Technol. Int. Tokyo*, **3**, 366–369 (1997).
106) Suzuki T., Yamaguchi M., *Food Sci. Technol. Res.*, **9**, 87–90 (2003).
107) Suzuki T., Yamaguchi M., *Food Sci. Technol. Res.*, **5**, 304–307 (1999).
108) Suzuki T., Yamaguchi M., *J. Health Sci.*, **50**, 483–490 (2004).
109) Suzuki T., Yamaguchi M., *Food Sci. Technol. Res.*, **5**, 61–63 (1999).
110) Yamaguchi M., Ma Z. J., Suzuki T., *J. Health Sci.*, **49**, 123–128 (2003).
111) Yamaguchi M., Hachiya S., Hiratsuka S., Suzuki T., *J. Health Sci.*, **47**, 533–538 (2001).
112) Uchiyama S., Yamaguchi M., *J. Health Sci.*, **48**, 148–153 (2002).
113) Uchiyama S., Yamaguchi M., *J. Health Sci.*, **48**, 154–160 (2002).
114) Uchiyama S., Hashizume M., Hokari Y., Nakagawa T., Igarashi A., Yamaguchi M., *J. Health Sci.*, **50**, 634–639 (2004).
115) Uchiyama S., Yamaguchi M., *J. Health Sci.*, **48**, 325–330 (2002).
116) Uchiyama S., Yamaguchi M., *J. Health Sci.*, **49**, 149–155 (2003).
117) Bouillon R., *Calcif. Tissue Int.*, **49**, 155–160 (1991).
118) Levin M. E., Boissesu V. C., Avioli L. V., *N. Engl. J. Med.*, **294**, 241–245 (1976).
119) Yamaguchi M., Hamamoto R., Uchiyama S., Ichiyama K., Hashimoto K., *J. Health Sci.*, **52**, 43–49 (2006).
120) Hamamoto R., Ishiyama K., Yamaguchi M., *J. Health Sci.*, **52**, 1–8 (2006).
121) Hidaka S., Okamoto Y., Uchiyama S., Nakatsuma A., Hashimoto K., Ohnishi S. T., Yamaguchi M., *Evidence-based Complem. Altern. Med.*, **3**, 339–348 (2006).
122) Hamamoto T., Ishiyama K., Hashimoto K., Yamaguchi M., *J. Health Sci.*, **52**, 607–612 (2006).
123) Lai Y. L., Yamaguchi M., *Mol. Cell. Biochem.*, (2006) (in press).
124) Lai Y. L., Yamaguchi M., *J. Health Sci.*, **52**, 308–312 (2006).