— Review —

β-Cryptoxanthin and Bone Metabolism: The Preventive Role in Osteoporosis

Masayoshi Yamaguchi*

Division of Endocrinology and Metabolism and Lipids, Department of Medicine, Emory University School of Medicine, 1639 Pierce Drive, 1305 WMRB, Atlanta, Georgia 30322–0001, U.S.A.

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Bone loss with aging induces osteoporosis. The most dramatic expression of the disease is represented by fractures of the proximal femur. Pharmacologic and nutritional factors may play a role in the prevention of bone loss with aging. β-Cryptoxanthin, a kind of carotenoid, is abundant in Satsuma mandarin orange (*Citrus unshiu* MARC.). Amoung various carotenoids including β-cryptoxanthin, lutein, lycopene, β-carotene, astaxanthin, and rutin, β-cryptoxanthin has been found to have a unique anabolic effect on bone calcification *in vitro*. Hesperidin, which is contained in Satsuma mandarin orange, did not have an anabolic effect on bone calcification *in vitro*. β-Cryptoxanthin has stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoclastic bone resorption *in vitro*, thereby increasing bone mass. β-Cryptoxanthin has an effect on the gene expression of various proteins which are related to osteoblastic bone formation and mineralization *in vitro*. β-Cryptoxanthin has inhibitory effects on enzyme activity which is related to osteoclastic bone resororption, and the carotenoid induces apoptosis of mature osteoclastic cells *in vitro*. Oral administration of β-cryptoxanthin has been shown to have the anabolic effects on bone components in young and aged rats, and the administration has the preventive effects on bone loss in streptozotocin-diabetic rats and ovariectomized rats *in vivo.* Moreover, the intake of β-cryptoxanthin-reinforced juice for longer periods has been shown to have both stimulatory effects on bone formation and inhibitory effects on bone resorption in healthy human or postmenopausal women in evaluating with serum biochemical markers of bone metabolism *in vivo*. Thus the intake of dietary β-cryptoxanthin may have a preventive effect on osteoporosis due to stimulating bone formation and due to inhibiting bone resorption. Moreover, epidemiological studies suggest the potential role of β-cryptoxanthin as a sustainable nutritional approach to improving bone health of human subjects. β-Cryptoxanthin is an important food factor in maintaining bone healthy and in preventing osteoporosis.

Key words —— β-cryptoxanthin, osteoblastic bone formation, osteoclastic bone resorption, osteoporosis

INTRODUCTION

Bone mass decreases with increasing age. The decrease in bone mass is due to increased bone resorption and decreased bone formation. Osteoclasts induce bone resorption, and osteoblasts stimulate bone formation. Ovarian hormone deficiency at menopause in women stimulates bone loss. A further possible cause is the deterioration of osteoblastic cell function with increasing age. Osteoporosis is induced with decrease in bone mass as shown in Fig. 1, and it is widely recognized as a major public health problem. The most dramatic expression of the disease is represented by fractures of the proximal femur for which the number increases as the population ages.[1, 2)]

Pharmacological and nutritional factors may have the potential effect to prevent bone loss with increasing age. Nutritional factors may be especially important in the prevention of osteoporosis.[3–5)] Chemical factors in food and plants can help to prevent bone loss with increasing age but these factors are poorly understood.

Our studies have shown that isoflavones, which are contained in soybeans, or menaquinone-7 (vitamin K_2), which is abundant in fermented soybeans, have been demonstrated to increasing bone mass due to stimulating osteoblastic bone formation and

*To whom correspondence should be addressed: Division of Endocrinology and Metabolism and Lipids, Department of Medicine, Emory University School of Medicine, 1639 Pierce Drive, 1305 WMRB, Atlanta, Georgia 30322–0001, U.S.A. Tel.: +1-404-727-2598; Fax: +1-404-727-1300; E-mail: yamamasa1155@yahoo.co.jp

to inhibiting osteoclastic bone resorption *in vitro* [Refs. 3), 4) in Review]. The supplementation of these food factors may have preventive effects on bone loss induced in animal model of osteoporosis and in human subjects.

Other food and plant factors (including flavonoid,[6] *p*-hydroxycinnamic acid,[7,8] the extracts of *Sargassum horneri*,[9,10] bee pollen of *Cistus ladaniferus*,[11,12] or wasabi leafstalk[13,14]) have also shown to increase bone mass in the femoral tissues of rats *in vitro* and *in vivo*. Food chemical factors thus play an important role in bone health and prevention of bone loss.

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.[15] The effects of carotenoids on bone metabolism, however, have not been fully clarified. Carotenoids are present in fruit and vegetables.

β-Cryptoxanthin is a kind of carotenoid which is abundant in Satsuma mandarin orange (*Citrus unshiu* MARC.). The chemical structure of β-cryptoxanthin is shown in Fig. 2. The biological function of β-cryptoxanthin in animal and human, however, was not clarified thus far. We found that β-cryptoxanthin has a unique anabolic effect on bone metabolism;[16,17] such an effect was not seen by lutein, lycopene, or astaxanthin, which is other carotenoids, and flavonoid rutin (quercetin-3-rutinoside). This was the first time finding.

This review introduces the cellular mechanism by which β-cryptoxanthin increases bone mass and its role in the prevention of osteoporosis.

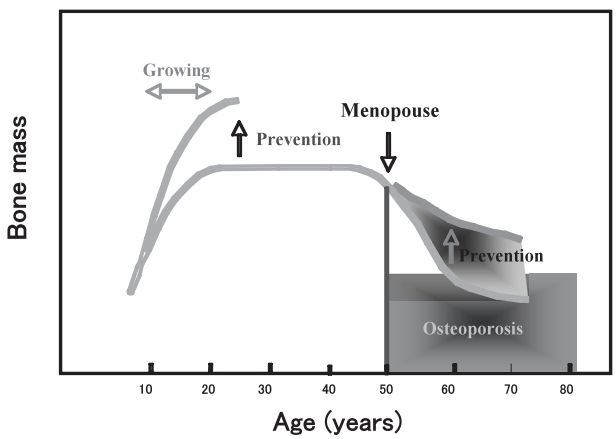

Fig. 1. Change in Bone Mass with Increasing Age

Bone mass increases with growing and reaches to peak in the range of age between twenty and twenty-five years. Then bone mass gradually decreases with aging. In postomenopausal women, bone mass decreases dramatically, and it induces osteoporosis. It is important to prevent the decrease in bone mass with increasing age.

BASIC ASPECTS ON REGULATION OF BONE METABOLISM

Bone contains over 98% of total body calcium. Bone metabolism is regulated by the functions of osteoblasts and osteoclasts, which are major cells in bone tissues,[18–20] as shown in Fig. 3.

Osteoclasts, which develop from hematopoietic progenitors, are recruited to the site and excavate the calcified matrix. Then, the cavity is refilled by osteoblasts via a process that occurs in three distinct phases: initiation, progression, and termination.

In the physiologic process of bone turnover, a resorptive stimulus firstly triggers recruitment of osteoclasts to a site on the bone surface. This is followed by active resorption by osteoclasts, after which cells withdraw from the bone surface and mononuclear phagocytic cells appear on the newly resorbed surface. These cells are then followed by young osteoblasts, which begin the bone formation phase.

During the initiation phase, a team of osteoblasts arising from local mesenchymal stem cells assembles at the bottom of the cavity and bone formation begins. After the resorbed lacunar pit is filled with new osteoid, osteoblasts become flatter and less active, with the final newly remodeled bone surface lined by flat lining cells. Remodeling of

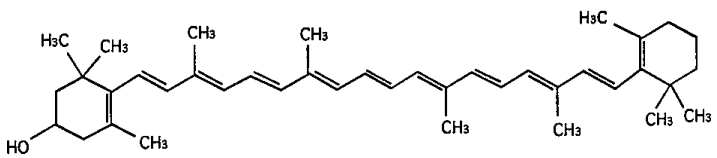

Fig. 2. Chemical Structure of β-Cryptoxanthin

The molecular weight of this compound is 552. β-Cryptoxanthin is a kind of carotenoid which is abundant in Satsuma mandarin (*Citrus unshiu* MARC.).

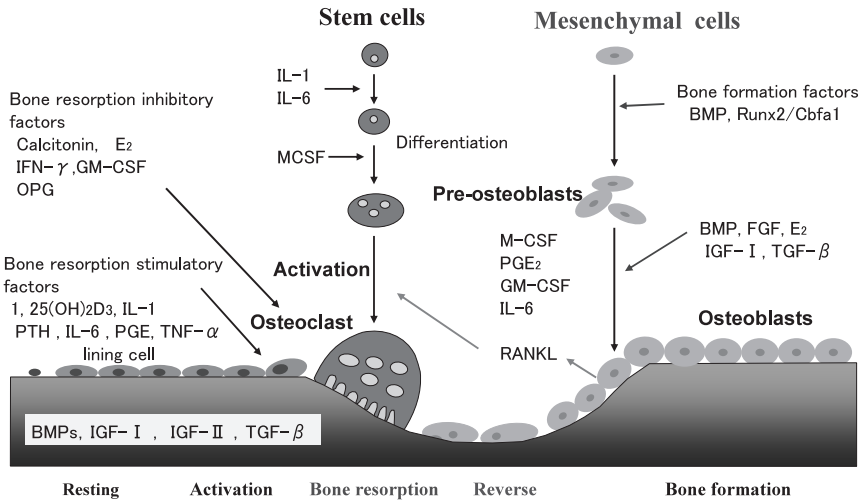

Fig. 3. Regulatory Mechanism in Bone Remodeling

Bone metabolism is regulated by osteoclasts and osteoblasts. Osteoclasts, which stimulate bone resorption, are generated from stem cells by interleukin-1 (IL-1), IL-6, M-CSF, and other factors. Osteoclastogenesis is mediated through RANKL produced from osteoblasts by various bone resorption stimulatory factors. Osteoblasts, which stimulate bone formation and mineralization, are differentiated from mesenchymal cells by various bone formation factors. After bone formation and calcification, osteoblasts are changed to osteocytes in bone matrix.

cancellous bone begins with the retraction of lining cells that cover the bone surface.[18)]

As bone formation progresses, some osteoblasts are entombed within the matrix as osteocytes but the majority dies by apoptosis. Bone formation terminates when the cavity has been refilled, at which time the few osteoblasts that remain become the flat lining cells that cover the quiescent surfaces of bone. Once formed, few osteocytes die. Their viability is likely maintained by physiological levels of mechanical stimulation. When mechanical forces are reduced, for example in weightlessness, osteocytes die by apoptosis. This event appears to act as a beacon for osteoclast recruitment and generation of a new basic multicellular unit, which in turn replaces the old bone containing dead osteocytes with new bone containing viable osteocytes.

Bone acts as major storage site for gowth factors.[20)] Growth factors, which are produced by osteoblasts, diffuse into newly deposited osteoid and are stored in the bone matrx including isulin-like growth factors (IGF-I and II), transforming growth factor-β1 (TGF-β1), platelet-derived growth factor (PDGF), or bone morphologic protein (BMP). These bone-derived factors, which can be liberated during subsequent periods of bone resorption, act in an autocrine, paracrine, or delayed paracrine fashion in the local microenvironment of the bone surface.

It is this process of bone remodeling that makes bone unique among organs and tissues and that also adds so many levels of complexity, with respect to interactions along the remodeling sequence by systemic influences (hormones), stress action on trabecular and cortical systems (physical activity/weight bearing), growth factors and cytokines produced by the bone cells which act locally on their own cell types and on the other bone cell types, or factors that come from nearby cells present in the marrow tissues.

It is interested whether food factors have a role in the regulation of bone remodeling.

β-CRYPTOXANTHIN AND BONE METABOLISM

β-Cryptoxanthin Stimulates Bone Formation and Inhibits Bone Resorption in Tissue Culture *In Vitro*

Amoung of various carotenoid and flavonoids, β-cryptoxanthin has been shown to have a unique anabolic effect on bone calcification.[11, 16, 17)] 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 (cortical bone) and -metaphyseal (trabecular bone) tissues *in vitro*. 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. Astaxanthin and β-carotene (10^{-6}

or 10^{-5} M) did not have an effect on the femoral calcium contents. Myricetin, kaempferol, isorhamnetin, curcumin, or hesperidin (10^{-7} to 10^{-5} M) had no effect on bone calcium content in tissue cultures *in vitro*.[11] Quercetin significantly increased calcium content in femoral diaphyseal tissues but not metaphyseal tissues. Alkaline phosphatase participates in mineralization in bone tissues.[21] β-Cryptoxanthin had a unique anabolic effect on bone calcification *in vitro*. The effect of β-cryptoxanthin increasing bone components was completely prevented with cycloheximide, an inhibitor of protein synthesis, suggesting that the effect is needed newly protein synthesis.[17]

β-Cryptoxanthin has been shown to inhibit bone resorption in bone tissue cultures.[17] Parathyroid hormone (PTH) or prostaglandin E_2 (PGE_2), which is bone-resorbing factor, can stimulate osteoclastic bone resorption *in vitro*.[21–23] Culture with PTH or PGE_2 caused a significant decrease in calcium content in the diaphyseal and metaphyseal tissues.[17] This decrease was completely inhibited in the presence of β-cryptoxanthin (10^{-8} to 10^{-6} M).[17] Also, culture with β-cryptoxanthin completely inhibited the PTH- or PGE_2-induced increase in medium glucose consumption and lactic acid production by bone tissues.[17] β-Cryptoxanthin had inhibitory effects on bone resorption in tissue culture *in vitro*.

Thus β-cryptoxanthin was found to have stimulatory effect on bone formation and inhibitory effects on bone resorption in bone tissue culture *in vitro*.

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.[24] 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.

Cellular and Molecular Mechanisms of β-Cryptoxanthin Action in Osteoblastic Cells

The cellular and molecular mechanisms by which β-cryptoxanthin stimulates bone formation in bone tissues were examined using cloned osteoblastic cells *in vitro*. Osteoblastic MC3T3E1 cells were used. β-Cryptoxanthin was found to stimulate the proliferation of osteoblastic cells in subconfluent monolayers in a medium containing 10% fetal bovine serum.[25] Culture with β-cryptoxanthin also caused a significant increase in biochemical components (protein content, alkaline phosphatase activity, and DNA content) of osteoblastic cells.[25] This effect was significantly abolished in the presence of staurosporine, an inhibitor of protein kinase C, or PD98059, an inhibitor of mitosis activated protein kinase (MAP) kinase, although the effect of β-cryptoxanthin in increasing cellular biochemical components was not significantly prevented by dibucain, an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase.[25] The stimulatory effect of β-cryptoxanthin on osteoblastic cell components seems to be partly mediated through signaling factors of protein kinase C or MAP kinase in the cells.

Also, the effects of β-cryptoxanthin in increasing the biochemical components in osteoblastic cells are completely inhibited in the presence of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), an inhibitor of RNA polymerase II, suggesting that the carotenoid effect results from a stimulatory effect on transcriptional activity in osteoblastic cells.

The mineralization in osteoblastic cells has been shown to stimulate by prolonged culture with β-cryptoxanthin.[26] The stimulatory effect of β-cryptoxanthin on mineralization may result from the carotenoid-induced proliferation and differentiation of osteoblastic cells. Whether β-cryptoxanthin stimulates gene expression for proteins that involve in bone formation and mineralization in osteoblastic cells, is important.

Then the effect of β-cryptoxanthin on gene expression in osteoblastic cells using reverse transcription-polymerase chain reaction (RT-PCR) was examined. Culture with β-cryptoxanthin was found to stimulate the mRNA expression of IGF-I or TGF-β1 in osteoblastic cells.[25] This finding may support the view that β-cryptoxanthin has a stimulatory effect on transcriptional activity in osteoblastic cells. IGF-I or TGF-β1 is a bone growth factors produced from osteoblasts.[27,28] The stimulatory effect of β-cryptoxanthin on the proliferation of 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 mRNA expression of Runx2, α1(I) collagen, and alkaline phosphatase in osteoblastic MC3T3-E1 cells.[26] Runx2 (Cbfa1) is a member of the runt domain family of transcription factors and a master regulator of osteoblast differentiation.[29] α1(I) Collagen is a matrix protein that is related to bone formation and mineralization

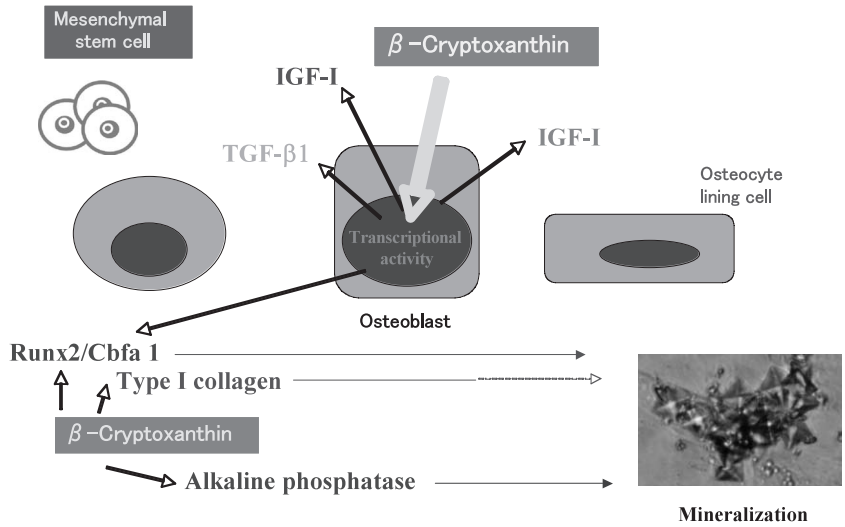

Fig. 4. β-Cryptoxanthin Stimulates Osteoblastic Bone Formation and Mineralization

β-Cryptoxanthin stimulates proliferation and differentiation of osteoblastic cells. The carotenoid stimulates gene expression of Runx2, type I collagen, alkaline phosphatase, IGF-I, or TGF-β1 that involves in differentiation and mineralization in osteoblastic cells. The increase in these protein molecules induces bone formation and mineralization.

in osteoblast lineage cells.[30] Alkaline phosphatase participates in the mineralization process in osteoblastic cells.[31] β-Cryptoxanthin has a stimulatory effect on the gene expression of for various proteins involved in osteoblasic bone formation. The effect of β-cryptoxanthin in stimulating mineralization in osteoblastic cells is summarized in Fig. 4.

The effects of β-cryptoxanthin in stimulating Runx2, α1(I) collagen, and alkaline phosphatase mRNA expression in osteoblastic MC3T3-E1 cells was found to prevent completely in the presence of DRB,[26] supporting the view that the carotenoid stimulates transcriptional activity in osteoblastic MC3T3-E1 cells.

Vitamin A (retinol) may be able to bind to nuclear receptors in cells. Retinol and β-carotene is shown to inhibit the proliferation of osteoblastic MC3T3-E1 cells as well as DNA synthesis of the cells, due to increasing alkaline phosphatase activity dose dependently (10^{-9} to 10^{-7} M).[32] 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 was also seen in the presence of vitamin A (10^{-6} M).[26] 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.[26] 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 retinoid X receptor in the nucleus of the cells.[26] 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. The mechanism of β-cryptoxanthin action in stimulating proliferation, differentiation, and mineralization in osteoblastic cells is summarized in Fig. 5.

Cellular and Molecular Mechanisms of β-Cryptoxanthin Action in Osteoclasts

Effect on Osteoclastogenesis: The receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) plays a pivotal role in osteoclastogenesis from bone marrow cells. RANKL expression is induced in osteoblastic cells and bone marrow stromal cells in response to osteoporotic factors, such as PTH, PGE_2, and 1,25-dihydroxyvitamin D_3 (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*.[33,34] The receptor protein RANK (receptor activator of NF-κB) is expressed on the surface of osteoclast progenitors.

β-Cryptoxanthin (10^{-8} to 10^{-6} M) was shown to have a potent inhibitory effect on osteoclast-like

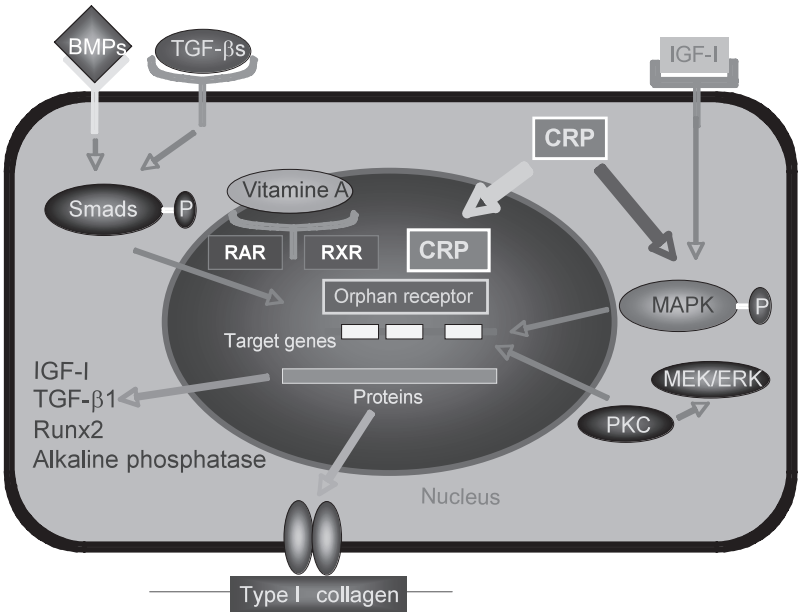

Fig. 5. The Cellular Mechanism by Which β-Cryptoxanthin Stimulates Bone Formation and Mineralization in Osteoblastic Cells

β-Cryptoxanthin (CRP) may bind to orphan receptors in the nucleus of osteoblastic cells, and it stimulates gene expression of bone formation-related proteins. CRP also stimulates nuclear transcriptional activity mediated through activation of PKC or MAPK in osteoblastic cells.

cell formation in mouse marrow culture *in vitro*.[35] The inhibitory effect of β-cryptoxanthin on osteoclast-like cell formation was seen at the later stage of osteoclast differentiation in bone marrow cultures. Culture with β-cryptoxanthin caused a marked inhibition of osteoblast-like cell formation induced in the presence of PTH, PGE_2, VD_3, lipopolysaccharide (LPS), or tumor necrosis factor-α (TNF-α). β-Cryptoxanthin also had a significant inhibitory effect on osteoclast-like cell formation induced by RANKL.[35] The inhibitory effect of β-cryptoxanthin was equal to that of 17 β-estradiol (E_2), calcitonin, genistein, and zinc sulfate, which can inhibit osteoclast-like cell formation induced by bone-resorbing factors.[35]

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 and c-Jun *N*-terminal kinase (JNK).[36, 37]

Protein kinase C family enzyme has a role in regulation of osteoclast formation and function potentially by participating in the extracellular signaling-regulated kinase (ERK) signaling pathway of M-CSF and RANKL.[38] Phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, significantly stimulated osteoclast-like cell formation in mouse marrow cultures, and the PMA-induced osteoclastogenesis is inhibited in the presence of β-cryptoxanthin.[35] Moreover, β-cryptoxanthin is found to have a significant inhibitory effect on dibutyryl cyclic adenosine monophosphate (DcAMP)-induced osteoclast-like cell formation in mouse marrow cultures.[35] It is assumed that activation of protein kinase C and protein kinase A pathways leads to increased RANKL expression, and that β-cryptoxanthin can inhibit protein kinase C- or protein kinase A-related RANKL expression in osteoclastogenesis.

The effect of β-cryptoxanthin in inhibiting osteoclastogenesis is summarized in Fig. 6.

Effect on Osteoclastic Function: The effects of β-cryptoxanthin on mature osteoclasts were also investigated.[38] M-CSF-dependent bone marrow macrophages were cultured in the presence of M-CSF and RANKL for 4 days.[38] The osteoclastic cells formed were further cultured in medium containing β-cryptoxanthin with or without M-CSF and RANKL for 24–72 hr. The number of osteoclastic cells were significantly decreased in culture with β-cryptoxanthin (10^{-7} or 10^{-6} M) in the presence or absence of M-CSF and RANKL for 72 hr. 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. These findings indicate that the carotenoid induces apoptotic cell death.

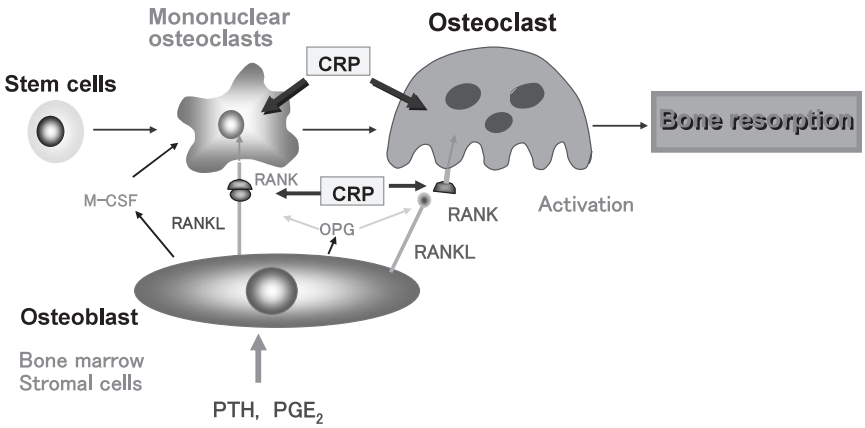

Fig. 6. β-Cryptoxanthin Inhibits Osteoclastogenesis in Bone Marrow Culture

β-Cryptoxanthin (CRP) inhibits osteoclast formation from mononuclear osteoclasts which are mediated through RANKL and RANK signaling in bone marrow culture systems. CRP also induces apoptotic cell death of mature osteoclasts. By such a cellular mechanism, CRP inhibits osteoclastic bone resorption. Whether CRP stimulates production of osteoprotegerin (OPG), which inhibits binding of RANKL to RANK receptor, in osteoblastic cells is unknown.

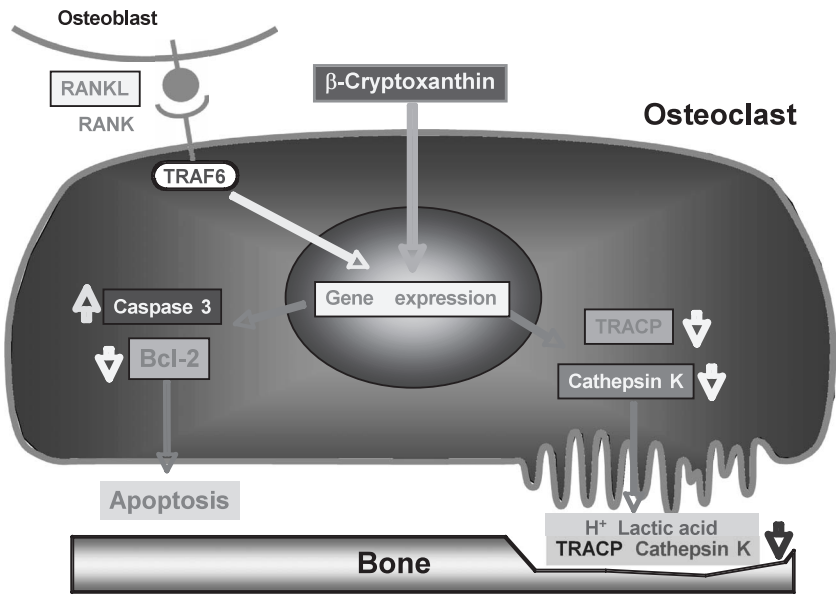

Fig. 7. The Cellular Mechanism by Which β-Cryptoxanthin Has Suppressive Effects on Mature Osteoclasts

β-Cryptoxanthin stimulates apoptotic cell death by stimulating the gene expression of caspase-3, an apotosis-inducing enzyme, and suppression of gene expression of Bcl-2, a rescue protein of apoptosis. Additionally, β-cryptoxanthin suppresses gene expression of TRACP and cathepsin K, which are bone resorption-related enzymes, and their enzyme activities in mature osteoclasts.

Apoptosis-related gene expression was determined using RT-PCR.[38] The expression of caspase-3 mRNA or apoptotic peptidase activating factor-1 (Apaf-1), which involves apoptosis, in osteoclastic cells was found to stimulate when cultured with β-cryptoxanthin in the presence or absence of M-CSF and RANKL.[38] β-Cryptoxanthin-induced apoptotic cell death is partly mediated through caspase-3 expression in osteoclastic cells.

The expression of B-cell CLL/lymphoma 2 (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.[38] However, v-akt murine thymoma viral oncogene homolog 1 (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, and it decreases TRACP and cathepsin K mRNA expressions in osteoclastic cells in the presence or absence of M-CSF and RANKL.[38] These findings suggest that β-cryptoxanthin can inhibit the enhancement of bone-resorbing activity in osteoclasts. β-Cryptoxanthin could inhibit various bone-resorbing factors-induced decrease in bone calcium content and increase in lactic acid production in rat femoral tisuue culture system *in vitro*.[13] Presumably, β-cryptoxanthin has inhibitory effects on the activation of mature osteoclasts.

β-Cryptoxanthin has been demonstrated to have stimulatory effects on apoptotic cell death due to activating gene expression of its related proteins. The carotenoid also has suppressive effects on TRACP activity and gene expression of enzymes that involve in bone-resorbing activity in osteoclastic cells. The action of β-cryptoxanthin in osteoclasts is summarized in Fig. 7.

PREVENTIVE ROLE OF β-CRYPTOXANTHIN IN OSTEOPOROSIS

As mentioned above, β-cryptoxanthin has been shown to have a stimulatory effect on osteoblastic bone formation and an inhibitory effect on osteoclastic bone resorption *in vitro*. The preventive effect of β-cryptoxanthin on osteoporosis was investigated using animal models.

Effect of β-Cryptoxanthin in Animal Models for Osteoporosis

Effects on Young and Aged Rats: The anabolic effect of β-cryptoxanthin on bone components in young and aged rats was examined.[39] β-Cryptoxanthin (10, 25 or 50 μg/100 g body weight) was orally administered once daily for 7 days to young male rats.[39] The administration of β-cryptoxanthin (25 or 50 μg/100 g body weight) caused a significant increase in calcium content, alkaline phosphatase activity, and DNA contents in the femoral-diaphyseal and -metaphyseal tissues. Such an effect is also observed in the femoral tissues of aged (50-week-old) female rats.[40] Alkaline phosphatase is an enzyme marker of osteoblasts, and the enzyme participates in bone mineralization.[41] DNA content in bone tissues is an index of the number of bone cells.[42] β-Cryptoxanthin has been shown to have an anabolic effect on bone components in rats *in vivo*.

Effect on Bone Loss Induced in Diabetic Rats: Whether β-cryptoxanthin has a preventive effect on bone loss in the pathophysiologic state was examined. Bone loss has been shown to induce in streoptozotocin (STZ)-diabetic rats.[43] 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 after the administration of β-cryptoxanthin (5 or 10 μg/100 g) for 14 days. 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 after the administration of β-cryptoxanthin (5 or 10 μg/100 g) for 14 days. Thus the intake of β-cryptoxanthin was found to have preventive effects on STZ-diabetic state and bone loss in STZ-diabetic rats.[43]

Effect on Bone Loss in Ovariectomized Rats: Bone loss is induced after ovriectomy (OVX), which is a model of postmenopausal osteoporosis. The effect of β-cryptoxanthin on OVX-induced bone loss was examined.[44] 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 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 after the administration of β-cryptoxanthin (5 or 10 μg/100 g). Moreover, OVX induced a significant decrease in bone biochemical components. These decreases were completely prevented after the administration of β-cryptoxanthin (5 or 10 μg/100 g). β-Cryptoxanthin was found to have preventive effects on OVX-induced bone loss *in vivo*.[44]

Effects of β-Cryptoxanthin in Healthy Individuals and Menopausal Women

The effects of β-cryptoxanthin on bone metabolism in human were investigated using serum bone metabolic markers. Serum bone-specific alkaline phosphatase and γ-carboxylated osteocalcin are bone metabolic markers of os-

teoblastic bone formation.[45,46] Serum bone TRACP and *N*-telopeptides of type I collagen are metabolic markers of osteoclastic bone resorption.[47,48]

The effects of prolonged intake of juice prepared from Satsuma mandarin (*Citrus unshiu* MARC.) containing β-cryptoxanthin on circulating biochemical markers of bone metabolism in subjects, including menopausal woman, were examined.[49–51]

Effect on Healthy Individuals: Twenty-one volunteers (10 males and 11 females) were divided into two groups of ten volunteers (5 males and 5 females) and eleven volunteers (5 males and 6 females), and each group was given sequentially juice (192 ml) containing two different contents of β-cryptoxanthin once a day for 28 or 56 days as follows: either regular juice with naturally occurring 802 μg β-cryptoxanthin/100 ml or a reinforced juice containing 1500 μg β-cryptoxanthin/100 ml.[49] The intake of regular juice for 28 or 56 days in healthy subjects caused a significant increase in serum γ-carboxylated osteocalcin concentration, and the 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 serum γ-carboxylated osteocalcin concentration and a corresponding decrease in serum bone TRACP activity and *N*-telopeptide of type I collagen. These findings suggest that the intake of β-cryptoxanthin reinforced juice has a stimulatory effect on osteoblastic bone formation and inhibitory effect on osteoclastic bone resorption in normal individuals.[49]

The serum β-cryptoxanthin concentration was significantly increased with the intake of regular juice for 56 days.[50] This increase was significantly enhanced after the intake of β-cryptoxanthin-reinforced juice. The intake of regular juice or of β-cryptoxanthin-reinforced juice for 56 days caused a significant increase in serum γ-carboxylated osteocalcin and a significant decrease in serum bone TRACP activity. A possible relationship between serum β-cryptoxanthin and circulating γ-carboxylated osteocalcin concentrations 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 concentation and circulating TRACP activity was observed.[50] This study shows that a relationship between serum β-cryptoxanthin and circulating bone metabolic markers is found in healthy individuals with the intake of juice containing β-cryptoxanthin.

Effect on Menopausal Women: Ninety volunteers, aged 27–65 years (19 men and 71 women), were enrolled in this study.[51] The seventy one females included 35 premenopausal women (ages, 27–50 years) and 36 menopausal women (ages, 46–65 years). Volunteers were divided into four groups; placebo juice without β-cryptoxanthin (5 men and 19 women), juice containing β-cryptoxanthin at 1.5 mg/200 ml of juice/day (4 men and 17 women), 3.0 mg/day (5 men and 17 women), and 6.0 mg/day (5 men and 18 women). Placebo or juice (200 ml) was ingested once a day for 28 or 56 days.

Serum β-cryptoxanthin concentrations were significantly increased after the intake of juice containing β-cryptoxanthin (1.5, 3.0, or 6.0 mg/day) for 28 or 56 days, and the increases were dose-dependent.[51] A significant increase in serum β-cryptoxanthin concentration was also observed at 28 days at the end of intake, indicating that the carotenoid is stable in the serum. Serum β-cryptoxanthin concentration was in the range of 4.20×10^{-7} M to 4.89×10^{-7} M in the placebo groups. The intake of juice reinforced with β-cryptoxanthin concentration at doses of 1.5, 3.0, or 6.0 mg/day significantly increased the serum concentration to 2.43×10^{-6}, 4.06×10^{-6}, or 5.38×10^{-6} M, respectively.[51] These increases were about 5- or 10-fold as compared with the value obtained before intake or after placebo intake. It has been reported that the serum concentration of β-cryptoxanthin increased due to the consumption of vegetable juice in women from 1.3×10^{-7} to 5.3×10^{-7} M.[24]

In ninety volunteers (aged 27–65 years), serum bone-specific alkaline phosphatase activity was significantly increased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 56 days as compared with the value obtained before intake.[51] γ-Carboxylated osteocalcin concentration was significantly increased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 28 or 56 days as compared with the value obtained before intake or after the intake of placebo juice.[51] Serum TRACP activity and type I collagen *N*-telopeptide concentration were significantly decreased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 28 or 56 days as compared with the value obtained before intake or after intake of placebo juice, and significant decreases were also seen after the intake of 1.5 mg/day

β-cryptoxanthin as compared with the value obtained before intake.[51)]

In menopausal women (36 volunteers), bone-specific alkaline phosphatase activity and γ-carboxylated osteocalcin concentration were significantly increased after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 56 days as compared with the value obtained after placebo intake.[51)] Also, this intake caused a significant decrease in bone TRACP activity and type I collagen *N*-telopeptide concentration. The prolonged intake of β-cryptoxanthin-reinforced juice has been demonstrated to have stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoclastic bone resorption in menopausal women.

Meanwhile, serum calcium, inorganic phosphorous, and parathyroid hormone (intact) were not changed after the intake of β-cryptoxanthin-containing juice for 28 or 56 days. Other serum biochemical findings were not changed after the intake of juice containing β-cryptoxanthin (3.0 or 6.0 mg/day) for 56 days. We confirmed the safety of β-cryptoxanthin in human.[51)]

As the mentioned above, the intake of juice reinforced with β-cryptoxanthin (3.0 or 6.0 mg/day) had a significant effect on circulating bone metabolic markers in men, premenopausal women, and menopausal women.[51)] This indicates that the effects of β-cryptoxanthin in stimulating bone formation and inhibiting bone resorption are present in both sexes. Interestingly, the intake of juice reinforced with β-cryptoxanthin (3.0 or 6.0 mg/day) was found to have effects on circulating bone metabolic markers in menopausal women, indicating that the supplementation of β-cryptoxanthin has preventive effects on bone loss due to osteoporosis in menopausal women. This preventive effect was obvious at a dose of β-cryptoxanthin of 3.0 mg/day in menopausal women. This dose may be suitable in the prevention of osteoporosis in human subjects.

Thus the intake of reinforced juice, which contains more β-cryptoxanthin than regular juice, has been demonstrated to have a preventive effect on bone loss that accompanies an increase in age.

EPIDEMIOLOGICAL EVIDENCE FOR ROLE OF β-CRYPTOXANTHIN IN BONE HEALTHY

On the based on our findings, it has been recently reported that epidemiological studies show that the intakes of fruit and vegetables containing β-cryptoxanthin may reduce the risk of osteoporosis.[52–54)]

The effect of dietary antioxidants on knee structure in a cohort of healthy, middle-aged subjects with no clinical knee osteoarthritis is examined.[52)] Two hundred and ninety-three healthy adults (mean age = 58.0 years) without knee pain or knee injury are selected from an existing community-based cohort. The intake of antioxidant vitamins and food sources by these individuals was estimated from a food frequency questionnaire at baseline. The cartilage volume, bone area, cartilage defects and bone marrow lesions were assessed approximately 10 years later using magnetic resonance imaging. Higher vitamin C intake was associated with a reduced risk of bone marrow lesions and with a reduction in the tibial plateau bone area. There was an inverse association between fruit intake and the tibial plateau bone area and between fruit intake and the risk of bone marrow lesions. Neither fruit intake nor vitamin C intake was significantly associated with the cartilage volume or cartilage defects. Lutein and zeaxanthin intake was associated with a decreased risk of cartilage defects, and vitamin E intake tended top be positively associated with the tibial plateau bone are only after adjusting for vitamin C intake. The β-cryptoxanthin intake was inversely associated with the tibial plateau bone area after adjusting for vitamin E intake. These observations suggest a beneficial effect of fruit consumption and vitamin C intake as they are associated with a reduction in bone size and the number of bone marrow lesions, both of which are important in the patogenesis of knee osteoarthritis.[52)]

Bone mineral density (BMD) in post-menopausal female subjects has been shown to associate with serum antioxidant carotenoids. A total of six hundred ninety-nine subjects (222 males and 477 females) who had received health examinations in the town of Mikkabi, Shizuoka Prefecture, Japan, participated in the study.[53)] Radial BMD was measured using dual-energy X-ray absorptiometry. The associations of serum carotenoid concentrations with the dadial BMD were evaluated cross-sectionally. In male and pre-menopausal female subjects, the six serum carotenoids were not associated with the radial BMD. On the other hand, in post-menopausal female subjects, serum β-cryptoxanthin and β-carotene were weakly but positively correlated with the radial BMD. After adjustment for confounders,

the odds ratio (OR) for the lowest quartile of BMD in the high groups of serum β-cryptoxanthin against the lowest quartile was 0.45 in post-menopausal female subjects. However, this association was not significant after further adjusting for intakes of minerals and vitamins. Antioxidant carotenoids, especially β-cryptoxanthin, significantly but partly associate with the radial BMD in post-menopausal female subjects.[53)]

Seasonal variation of serum α- and β-cryptoxanthin and 25-OH-vitamin D_3 in women with osteoporosis is examined.[54)] In six hundred fourty-four women with osteoporosis, serum β-cryptoxanthin and 25-OH-vitamin D_3 showed a weak but significant correlation and exhibited a complementary seasonal distribution.[54)] Dietary intake and serum levels of β-cryptoxanthin have been inversely related to different bone and joint disorders and *in vitro* and animal studies have shown that β-cryptoxanthin displays a unique anabolic effect on bone calcification. Due to the emerging role of β-cryptoxanthin in bone biology, this study aimed to assess the serum distribution and variability of β-cryptoxanthin and their potential relation to 25-OH-vitamin D_3 in women with osteoporosis.

Overall, significant seasonal variations were found for the three analyses and inter-individual variation was also high (60–73%). β-Cryptoxanthin and 25-OH-vitamin D_3 exhibited a marked complementary seasonal distribution in serum, with vitamin D displaying the highest values in summer and β-cryptoxanthin in winter.

Given the anabolic effect of β-cryptoxanthin on bone calcification and its complementary seasonal distribution with respect to 25-OH-vitamin D_3, the potential role of β-cryptoxanthin as a sustainable nutritional approach to improving bone health deserves to be further evaluated.[54)]

CONCLUSION

Amoung various carotinoids, β-cryptoxanthin has a unique anabolic effect on bone mass due to stimulating osteoblastic bone formation and inhibiting osteoclastic bone resorption, thereby increasing bone mass. β-Cryptoxanthin modulates gene expression of various proteins that involve in bone formation in osteoblasts and bone resorption in osteoclasts. The intake of dietary β-cryptoxanthin has been shown to have preventive effect on bone loss in animal models for osteoporosis and restorative effect on bone metabolism in menopausal women. The role of β-cryptoxanthin in bone healthy has been also shown in human subjects with epidemiological studies. β-Cryptoxanthin may have an important role in prevention of osteoporosis.

Epidemiological studies, moreover, demonstrates the anabolic effect of β-cryptoxanthin on bone calcification and its complementary seasonal distribution with respect to 25-OH-vitamin D_3, a classic hormonal factor of bone healthy in human subjects,[54)] suggesting the potential role of β-cryptoxanthin as a sustainable nutritional approach to improving bone health. The physiologic significance of β-cryptoxanthin in bone healthy, however, remains to be elucidated.

Whether the combination of nutritional factors exhibits an additive or synergistic effect on bone components has not been fully clarified. This knowledge may be important in preventing bone loss with increasing age. The anabolic effect of β-cryptoxanthin on osteoblastic bone formation has not been shown to enhance synergistically in the presence of genistein, menaquinone-7, VD_3, or E_2, which has a stimulatory effect on osteoblastic bone formation.[55,56)] The combination of β-cryptoxanthin and zinc at a lower concentration, however, is found to have a synergistic effect on osteoblastic bone formation[55–57)] and an additive suppressive effect on osteoclastic cell functions.[58)] Zinc has been shown to stimulate osteoblastic bone formation and inhibit osteoclastic bone resorption.[59–63)] The finding is interested in respect of the development of new supplement with the composition of food factors that reveal a potent-anabolic effect in prevention of osteoporosis. It also would be useful to identify some of the foods that contain higher levels of β-cryptoxanthin and zinc.

Additionally, the intake of β-cryptoxanthin with higher dose may have a pharmacologic role in therapy of osteoporosis. Clinical studies are needed in the development of new drug for osteoporosis therapy.

In conclusion, β-cryptoxanthin is a kind of carotenoid that has a potential effect in maintaining bone healthy and in preventing osteoporosis.

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