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Role of nutritional zinc in the prevention of osteoporosis

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Abstract Zinc is known as an essential nutritional factor in the growth of the human and animals. Bone growth retardation is a common finding in various conditions associated with dietary zinc deficiency. Bone zinc content has been shown to decrease in aging, skeletal unloading, and postmenopausal conditions, suggesting its role in bone disorder. Zinc has been demonstrated to have a stimulatory effect on osteoblastic bone formation and mineralization; the metal directly activates aminoacyl-tRNA synthetase, a rate-limiting enzyme at translational process of protein synthesis, in the cells, and it stimulates cellular protein synthesis. Zinc has been shown to stimulate gene expression of the transcription factors runt-related transcription factor 2 (Runx2) that is related to differentiation into osteoblastic cells. Moreover, zinc has been shown to inhibit osteoclastic bone resorption due to inhibiting osteoclast-like cell formation from bone marrow cells and stimulating apoptotic cell death of mature osteoclasts. Zinc has a suppressive effect on the receptor activator of nuclear factor (NF)-κB ligand (RANKL)-induced osteoclastogenesis. Zinc transporter has been shown to express in osteoblastic and osteoclastic cells. Zinc protein is involved in transcription. The intake of dietary zinc causes an increase in bone mass. β-Alanyl-L-histidinato zinc (AHZ) is a zinc compound, in which zinc is chelated to β-alanyl-L-histidine. The stimulatory effect of AHZ on bone formation is more intensive than that of zinc sulfate. Zinc acexamate has also been shown to have a potent-anabolic effect on bone. The oral administration of AHZ or zinc acexamate has the restorative effect on bone loss under various pathophysiologic conditions including aging, skeletal unloading, aluminum bone toxicity, calcium- and vitamin D-deficiency, adjuvant arthritis, estrogen deficiency, diabetes, and fracture healing. Zinc compounds may be designed as new supplementation factor in the prevention and therapy of osteoporosis.

Keywords Zinc · Bone formation · Bone resorption · Osteoporosis

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Introduction

Zinc, a nutritional trace element, is essential for the growth of human and animals [1–3]. Zinc is required for the growth, development, and maintenance of healthy bones. Bone growth retardation is a common finding in various conditions associated with zinc deficiency [4–8]. In Iranian schoolboys at year 1961, zinc supplementation was first found to restore both skeletal growth and maturation [9]. Zinc deficiency is associated with many kinds of skeletal abnormalities in fetal and postnatal development. Zinc may play a physiologically important role in bone homeostasis.

Skeleton contains a large proportion of the total body burden of zinc [10]. Bone zinc has been shown to be concentrated in the layer of osteoid prior to calcification [11]. Zinc can be mobilized by conditions such as calcium deficiency in the pregnant rats [12]. Early mobilization of maternal bone provided sufficient zinc prevents fetal malformation, and most fetal zinc is derived from maternal muscle mobilized later in the calcium-deficient pregnancy [13].

Bone appears to act as a zinc sink. Zinc is released during skeletal breakdown, and it is mostly reincorporated

into the skeleton [14, 15]. In human, the vertebral calcium/zinc is inversely related to age, suggesting that skeletal zinc is conserved more than calcium in later life [16]. Zinc also occurs in the mineral component of bone, probably in hydroxyapatite [14, 17]; it may be complex with fluoride; and both zinc and the zinc-fluoride complex may improve the crystallinity of apatite [18].

Osteoporotic patients have been shown to have lower levels of skeletal zinc than control [19]. In postmenopausal women, urinary zinc has been suggested as a marker of bone resorption, since women with osteoporosis excrete over than 800 μg zinc/g creatinine in urine [10]. The supplements of trace minerals with or without calcium in postmenopausal women have been shown to have beneficial effects on bone density [20].

Zinc may play a physiologically important role in bone. The mechanism of action of zinc on osteoblastic and osteoclastic cells that regulate bone homeostasis, however, has been poorly defined. In recent years, there is growing evidence that zinc has an important role in the regulation of bone homeostasis. Zinc has been demonstrated to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption, thereby increasing bone mass. Supplementation of nutritional zinc has been shown to have preventive and therapeutic effects on bone loss that is induced in various bone disorders.

This review has been written to outline the recent advances that have been made concerning the role of zinc in the regulation of bone homeostasis and in the prevention and therapy of osteoporosis.

Regulation of bone homeostasis

Bone contains over 98% of total body calcium. Bone homeostasis is regulated with the functions of osteoblasts and osteoclasts, which are major cells in bone tissue [21–23]. In the physiologic process of bone turnover, a resorptive stimulus first triggers recruitment of osteoclasts to a site on the bone surface. 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. 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.

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.

The process of bone remodeling is regulated with respect to the interactions along the remodeling sequence by systemic influences (hormones), the stress action on trabecular and cortical systems with physical activity and weight bearing, various growth factors produced from the bone cells which act locally on the cells, or other factors that come from nearby cells present in the marrow tissues.

Bone acts as major storage site for growth factors [23]. Growth factors, which are produced by osteoblasts, diffuse into newly deposited osteoid and are stored in the bone matrix including insulin-like growth factors (IGF-I and II), transforming growth factor-β1 (TGF-β1), or platelet-derived growth factor (PDGF). 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.

Nutritional zinc has anabolic effect on bone

Zinc has an anabolic effect on bone metabolism in vivo and in vitro. The administration of zinc sulfate (5 and 10 mg Zn/kg body weight) for 3 days produced dose dependent increases in the contents of zinc, deoxyribonucleic acid (DNA), collagen and calcium, and the activity of alkaline phosphatase in the femoral diaphysis (cortical bone) of weanling rats [24]. Alkaline phosphatase is related to bone mineralization in osteoblasts. Collagen is a main bone matrix protein that is produced in osteoblasts. DNA content in bone tissues is a marker of the number of bone cells, including osteoblasts, osteoclasts, and osteocytes. Zinc accumulated in the bone tissues may first cause the activation of alkaline phosphatase and the stimulation of collagen synthesis in osteoblasts, which are involved in bone mineralization and calcification.

The anabolic effect on bone is not seen in other essential trace metals [24–26]. The oral administration of chromium (III), cobalt, copper, manganese, nickel, and selenium (15.3 μmol as metal/kg) for 3 days caused a significant increase in the diaphyseal alkaline phosphatase activity and DNA content, while the doses of 153 μmol/kg clearly decreased the enzyme activity. The dose of germanium (306 μmol/kg) caused a significant decrease in the diaphyseal alkaline phosphatase activity and DNA content. The

supplementation with copper in adequate copper nutrition does not have anabolic effects on bone components [27]. The oral administration of zinc with the dose of 3,060 μmol/kg produced an increases in both alkaline phosphatase activity and DNA content in the diaphyseal tissues. Zinc has the lowest toxicity for bone metabolism as compared with other trace metals.

Zinc has a stimulatory effect on bone formation in tissue culture system in vitro. Calvaria were removed from weanling rats, and it cultured for a period up to 96 h in Dulbecco's Modified Eagle Medium [28]. Zinc uptake by bone tissues was significantly increased in culture with zinc. Bone calcium content was significantly increased in the presence of 10^{-4} M zinc. Physiological zinc concentration in serum was in the range of 10^{-4} to 4×10^{-4} M. Bone alkaline phosphatase and ATPase activities were increased in the presence of zinc (10^{-6} to 10^{-3} M), whereas it did not change significantly the activities of pyrophosphatase, acid phosphatase, and *β-N*-acetylglucosaminidase. Bone collagen content was raised in the presence of zinc. The effect of zinc in increasing bone alkaline phosphatase activity and collagen content has been shown to prevent after the administration of an inhibitor of protein synthesis, suggesting that bone protein synthesis is a necessary component of this response.

The first step in the biosynthesis of protein involve the enzymatic activation of the amino acids with adenosine triphosphate, followed by the transfer of the amino acids to amino acid-specific ribonucleic acids (RNA). The presence of zinc in the culture medium has been found to induce a significant increase in the incorporation of [^{3}H]-leucine into the acid-insoluble residues of bone tissue [29]. The activity of [^{3}H] leucyl-tRNA synthetase in the 105,000 g supernatant fraction (cytosol) of the bone homogenate was increased about twofold after culture with zinc [30]. Zinc has a direct stimulatory effect on protein synthesis at the translational level in bone cells in vitro.

Endogenous zinc in bone tissues has also been shown to play an essential role on bone protein synthesis in the culture [31]. When the calvaria were cultured for 24 h in a medium containing dipicolinate (10^{-6} to 10^{-3} M), a chelator of zinc, bone zinc content was decreased after culture with dipicolinate [31]. In this case, bone alkaline phosphatase activity was decreased by about 40% of untreated bone enzyme activity. The decreased alkaline phosphatase activity was markedly increased in the presence of 10^{-4} M zinc (about 2.5-fold of control value). The effect of zinc was completely blocked after culture with protein synthesis inhibitor. Thus, bone endogenous zinc plays a role in the stimulation of protein synthesis at the translational process in bone cells. Zinc has been shown to increase many constitutive proteins in bone tissues in vitro [32].

Zinc has been shown to enhance the anabolic effect of vitamin D_3 on bone components in the femur of weanling rats in vivo [33]. The administration of vitamin D_3 (10 μg/kg) or zinc (10 mg Zn/kg) produced a significant increase in bone alkaline phosphatase activity and DNA content. The increase in alkaline phosphatase activity was additionally enhanced by the simultaneous administration of vitamin D_3 and zinc. The increase in bone DNA content was markedly (about fourfold) enhanced by both treatments. These increases were prevented after the administration of cycloheximide, an inhibitor of protein synthesis [33, 34]. The effect of 1,25-dihydroxyvitamin D_3 (0.5 and 1.5 μg/kg) in increasing bone alkaline phosphatase activity and DNA content was synergistically enhanced after the simultaneous treatment with zinc [34]. The receptors for 1,25-dihydroxyvitamin D_3 are shown to have two zinc fingers at the site of interaction with DNA [35]. Zinc may potentiate the interaction of the 1,25-dihydroxyvitamin D_3-receptor with DNA at that site. These findings suggest that the combination of vitamin D_3 and zinc has a synergistic effect on the stimulation of bone growth and mineralization in rats in vivo.

Zinc has also been shown to modulate hormonal effect on bone formation and calcification in bone tissue culture in vitro. The presence of 1,25-dihydroxyvitamin D_3 produced a significant increase in alkaline phosphatase activity, DNA, and calcium contents in tissue culture with rat calvaria [36]. The anabolic effect of estrogen (17*β*-estradiol) on bone components in tissue culture was significantly enhanced after culture with zinc sulfate [37]. The effect was abolished with cycloheximide. Zinc modulates anabolic effect of 1,25-dihydroxyvitamin D_3 or estrogen on bone metabolism in vitro. Zinc has been reported to increase the activity of 1,25-dihydroxyvitamin D_3-dependent promoters in osteoblastic cells [38].

Zinc stimulates osteoblastic bone formation

The cellular mechanism of zinc action in stimulating bone formation has been demonstrated in osteoblastic MC3T3-E1 cells. The proliferation of osteoblastic cells was stimulated after culture with zinc compound (zinc sulfate or zinc-chelating dipeptide) with or without fetal bovine serum [39]. Culture with zinc increased DNA content in the cells. Zinc-induced increase in cell proliferation and DNA content was prevented after culture with cycloheximide. The effect of zinc in stimulating cell proliferation may be mediated through protein synthesis [39]. Zinc has been shown to stimulate cell differentiation of osteoblastic cells [40]. Culture with zinc (10^{-7} to 10^{-5} M) produced a remarkable increase in alkaline phosphatase activity and protein concentration in osteoblastic cells. These

increases were also seen with the prolonged cultivation (12–21 days). The effect of zinc was completely abolished after culture with cycloheximide.

Protein components in osteoblastic MC3T3-E1 cells cultured with zinc have been characterized [41]. The homogenate of cells was analyzed with SDS-polyacrylamide gel electrophoresis. Culture with zinc caused an appreciable increase in many protein components in the cells. Especially, the 66 and 44 kDa proteins, which are the major components from control cells, were clearly found to increase in the presence of zinc compound. Moreover, the concentrations of osteocalcin, IGF-I, or TGF-β1 in the culture medium secreted from osteoblastic cells were markedly increased after culture with zinc. Zinc has been found to increase production of bone growth factors and bone matrix protein, which are involved in the stimulation of bone formation and mineralization in osteoblastic cells [41].

The effect of IGF-I in increasing protein concentration, DNA content, and cell number in the cells was markedly enhanced in the presence of zinc [42]. The cellular alkaline phosphatase activity was synergistically increased in the presence of both IGF-I and zinc. Such an effect was not seen after culture with both insulin and zinc. The enhancement of IGF-I's anabolic effect after culture with zinc has been shown to mediate through signaling pathway of protein kinase C and protein phosphatase in osteoblastic cells.

Culture with zinc sulfate has also been shown to increase protein tyrosine phosphatase activity in the cells [43]. The effect of IGF-I in increasing the enzyme activity was enhanced after culture with zinc [43]. Such an effect was not seen with parathyroid hormone (PTH). Zinc modulates the anabolic effect of IGF-I on protein tyrosine phosphatase activity and cell proliferation. Likewise, the cell proliferative effect of estrogen in osteoblastic cells has also been enhanced after culture with zinc compound.

The clarification of a molecular mechanism by which zinc stimulates bone protein synthesis has been attempted. Aminoacyl-tRNA synthetase is an enzyme that synthesizes aminoacyl-tRNA. Zinc has been found to activate [^{3}H]-leucyl-tRNA synthetase in the homogenate of osteoblastic cells [44].

Zinc has also been demonstrated to stimulate DNA synthesis in the homogenate of osteoblastic cells in vitro [45]. The culture with zinc compound clearly stimulated DNA synthesis in the homogenate of osteoblastic cells, when it was estimated with the incorporation of [^{3}H] deoxythimidine 5′-triphosphate into the DNA in the homogenate. The effect of zinc was completely abolished after culture with cycloheximide, suggesting that the action of zinc is based on newly synthesized protein components. The effect of zinc in stimulating DNA synthesis may mainly result from protein synthesis. Also, it is possible that zinc has an effect on the process of DNA synthesis that is involved in the direct activation of DNA polymerase, which is a zinc enzyme.

Zinc has been shown to stimulate the expression of transcription factors runt-related transcription factor 2 (Runx2) mRNA, a transcription factor, which is related to the differentiation to pre-osteoblastic cells [46].

As mentioned above, the mechanism of zinc action in stimulating osteoblastic bone formation and mineralization has been summarized in Fig. 1. Zinc stimulates cell proliferation, cell differentiation, and mineralization in osteoblasts, thereby promoting bone formation. Molecular mechanism of zinc action may be to stimulate gene expression of various proteins including Runx2/Cbfa1 (Core binding factor alpha1), type I collagen, alkaline phosphatase, and osteocalcin in the cells. Also, zinc increases production of IGF-I and TGF-β1 in the cells.

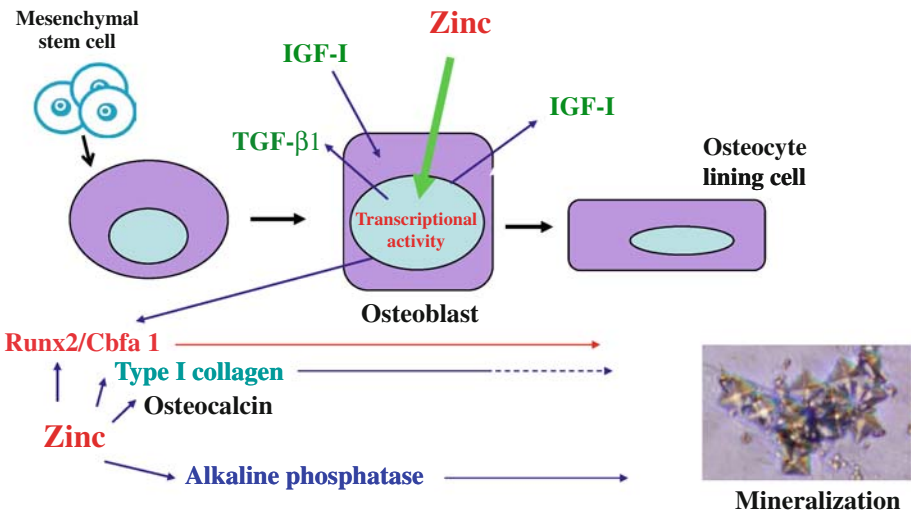

Fig. 1 Zinc stimulates cell differentiation, cell proliferation, and mineralization in osteoblasts. Zinc stimulates gene expression of various proteins including Runx2/Cbfa1 (transcription factor for differentiation into osteoclastic cells), type I collagen, alkaline phosphatase, and osteocalcin in the cells. Zinc also increases production of IGF-I and TGF-β1 in the cells. Zinc enhances protein synthesis due to activating aminoacyl-tRNA synthetase, a rate-limiting enzyme at translational process, in osteoblastic cells

Moreover, zinc enhances protein synthesis due to activating aminoacyl-tRNA synthetase, a rate-limiting enzyme at translational process, in osteoblastic cells. Thus, zinc has a potent stimulatory effect on osteoblastic bone formation.

Interestingly, the effect of IGF-I or estrogen on cell proliferation has been shown to enhance by zinc. The molecular mechanism of zinc action on cell nuclear events remains to be elucidated.

Zinc suppresses osteoclastic bone resorption

Zinc has an inhibitory effect on bone resorption [47]. Calvaria from weanling rats were cultured for the periods of up to 48 h in a medium containing various bone-resorbing factors [PTH, prostaglandin E_2 (PGE_2), interleukin-1α (IL-1α), and lipopolysaccharide]. These factors caused a significant decrease in bone calcium content. The decrease in bone calcium content was completely inhibited after culture with zinc compound [48]. Also, zinc compound completely inhibited the PTH or IL-1α induced increases in medium glucose consumption and lactic acid production by bone tissues [47]. The inhibitory effect of zinc compound on bone resorption was not seen after culture with dipicolinate, a chelator of zinc. Thus, zinc has been shown to have an inhibitory effect on bone resorption in tissue culture system in vitro.

PGE_2 is secreted from osteoblasts. PTH or IL-1α caused a remarkable elevation of PGE_2 production in osteoblasts in vitro [48]. Culture with zinc compound did not have an effect on PGE_2 production in osteoblasts [48], indicating that the effect of zinc may not mediated through suppression of PGE_2 production in osteoblasts.

Bone-resorbing cells, osteoclasts, are formed by differentiation of bone marrow cells. Zinc has an inhibitory effect on osteoclast-like cell formation in mouse marrow culture in vitro [49]. The bone marrow cells were cultured for 7 days in a medium containing bone-resorbing agent. Osteoclast-like cell formation was estimated staining for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts. The presence of 1,25-dihydroxyvitamin D_3, PTH, IL-1α, or PGE_2 induced a remarkable increase in osteoclast-like multinucleated cells. These increases were inhibited after culture with zinc (10^{-8} to 10^{-6} M). The inhibitory effect of zinc compound was equal in comparison with the effect of other anti-bone resorbing agents (calcitonin, 17β-estradiol, or acetazolamide) on osteoclast-like cell formation in mouse marrow culture. When osteoclast isolated from rat femoral-diaphyseal tissues were cultured for 24 h in the presence of zinc, the metal did not have an effect on lysosomal enzyme activity (acid phosphatase or β-glucuronidase) in the cells [50]. Culture with zinc caused apoptotic cell death of mature osteoclast-like cells isolated in rat femoral tissues [50]. Zinc has been shown to have suppressive effects on osteoclastogenesis and osteoclastic cell death that are generated with differentiation of bone marrow cells.

The mechanism of zinc action in inhibiting the PTH-induced osteoclast-like cell formation in mouse marrow culture system in vitro has been shown [51]. The effect of zinc in inhibiting PTH-induced osteoclast-like cell formation was clearly seen in the presence or absence of theophylline. However, zinc did not inhibit the stimulatory effect of dibutyryl cyclic AMP on osteoclast-like cell formation. The stimulatory effect of PTH on osteoclast-like cell formation was clearly weakened (about 50%) in the presence of EGTA or dibucaine, a regulatory factor of intracellular Ca^{2+} signaling. Phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator, stimulated osteoclast-like cell formation. The effect of PMA was inhibited after cultured with zinc. However, the inhibitory effect of zinc was not seen in the presence of both PTH and PMA. These findings support the view that zinc inhibits PTH-stimulated osteoclast-like cell formation mediated through the Ca^{2+}-dependent activation of protein kinase C.

Receptor activator of NF-κB ligand (RANKL) plays a pivotal role in the development of osteoclasts from pre-osteoclasts [52, 53]. RANKL is secreted from osteoblasts. RANKL is a member of the tumor necrosis factor (TNF) superfamily, which was originally identified as T-cell-derived immunomodulatory cytokines [54]. RANKL is expressed in activated T cells and promotes the survival of dendritic cells by binding to its receptor RANK. RANKL/RANK pathway is essential for osteoclast differentiation [52, 53]. RANKL is expressed in osteoblastic cells and bone marrow stromal cells in response to osteotropic factors. The combined treatment of hematopoietic cells with macrophage-colony stimulating factor (M-CSF) and the soluble from of RANKL (sRANKL) induces osteoclast differentiation in vitro [55]. The effect of RANKL was completely abrogated by adding a natural antagonist of RANKL, osteoprotegerin (OPG) [56], which is produced in osteoblastic cells. TNF receptor-associated factor (TRAF) family proteins are adaptor molecules that mediate intracellular signaling of various cytokine receptors including the TNF receptor superfamily and Toll/interleukin receptor (IL-1R) family [57]. TRAF6 binds to the membrane-proximal region of RANK and IL-1R-associated kinase, and is critically involved in the intracellular signal transduction including NF-κB and mitogen-activated protein kinase (MAPK) activation.

Zinc has been shown to have an inhibitory effect on RANKL-induced osteoclast-like cell formation in mouse marrow culture in the presence of M-CSF [58]. Zinc also inhibited TNFα-induced osteoclastogenesis [57]. TNFα is an autocrine factor in osteoclasts, promoting their

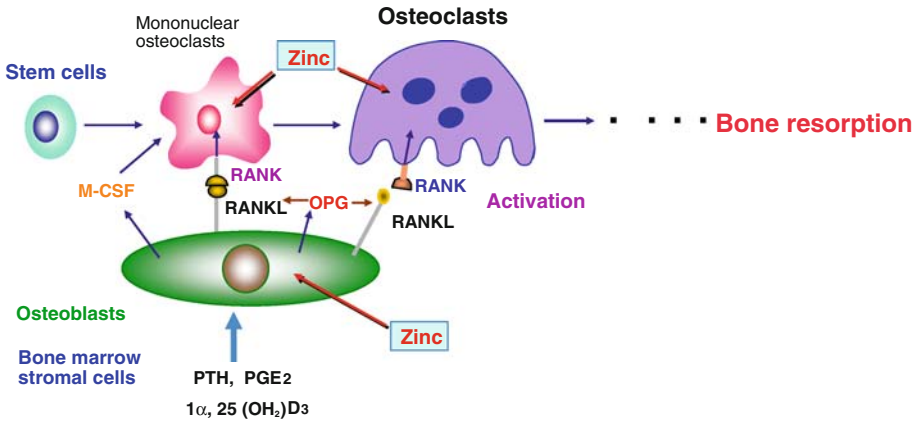

Fig. 2 Zinc inhibits osteoclastic bone resorption. Zinc suppresses osteoclast-like cell formation that is induced by various bone-resorbing factors in bone marrow culture. Zinc inhibits action of RANKL in pre-osteoclasts. Zinc also stimulates gene expression of OPG in osteoblastic cells that can inhibit the binding of RANKL to RANK receptors in pre-osteoclastic cells

differentiation, and mediates, at least in part, RANKL's induction of osteoclastogenesis [59]. The inhibitory effect of zinc on osteoclastogenesis may be partly involved in the suppressive effect on RANKL stimulation. In addition, zinc may inhibit signaling pathway that is related to RANKL stimulation in pre-osteoclasts.

The effect of zinc in inhibiting RANKL-induced osteoclastogenesis has been shown to abolish after culture with inhibitors of protein synthesis or transcription activity in mouse marrow cultures [58]. This finding suggests that the inhibitory effect of zinc on osteoclastogenesis is partly resulted from newly synthesized protein components that are involved in gene expression in mouse marrow culture.

Culture with zinc has been shown to have stimulatory effect on the expression of OPG mRNA in osteoblastic cells [46]. It is speculated that zinc stimulates the expression of RANKL inhibitor (including OPG) in osteoblasts, and that the metal induces a factor that can suppress osteoclast development in pre-osteoclasts.

As mentioned above, the cellular and molecular mechanism by which zinc inhibits osteoclastogenesis has been summarized in Fig. 2.

Role of zinc in bone growth

Zinc is an essential element for the growth of human and animals. Nutritional zinc is required for the growth, development, and maintenance of healthy bones. Bone growth retardation is a common finding in various conditions associated with zinc deficiency [4–8]. In fact, zinc has been demonstrated to stimulate bone growth in newborn rats supplied with lactation by maternal rats [60]. Newborn rats were killed between 1 and 35 days after birth. Increasing age caused a significant increase in zinc and calcium contents and alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues. The oral administration of zinc sulfate (20 mg Zn/kg body weight) with four times at 24-h intervals to maternal rats from 1 day after birth induced a significant increase in zinc, alkaline phosphatase activity, DNA and calcium contents in the femoral-diaphyseal and -metaphyseal tissues of newborn rats compared with those at 7 or 14 days old [60], indicating that the increase in bone components results from lactation with zinc-containing milk of maternal rats. Zinc plays a physiological role in the development of bone growth in newborn rats [60].

Endogenous zinc may be important in protein synthesis in the femoral-diaphyseal and -metaphyseal tissues of newborn rats [61]. Bone tissues were obtained at 1, 7, 14, 21, and 28 days after birth. Many protein molecules were found to be present in the bone tissues using SDS-polyacrylamide gel electrophoresis analysis. Bone protein synthesis activity was enhanced with increasing age, and reached a plateau 21 days after birth. Protein synthesis in the diaphyseal and metaphyseal tissues obtained from 7- or 14-day-old rats was significantly decreased after culture with a chelator of zinc ion [61]. This decrease was completely blocked after culture with addition of zinc. Zinc-induced increase in bone protein synthesis was completely prevented after culture with cycloheximide or actinomycin D. Thus, bone protein synthesis has been shown to enhance with increasing age of newborn rats, and endogenous zinc in bone tissues plays a physiologic role in the enhancement of protein synthesis in bone growth [61].

Zinc has been found to stimulate the productions of IGF-I and TGF-β1 in the bone tissues of newborn rats [62]. Femoral-diaphyseal and -metaphyseal tissues were obtained at 1, 7, 14, 21, and 28 days after birth of newborn rats, and cultured for 24 h in serum-free medium. Protein concentration in the culture medium was significantly increased when cultured bone tissues from newborn rats with increasing age. Medium IGF-I or TGF-β1 concentrations were gradually reduced with increasing age after birth. The presence of zinc sulfate (10^{-5} or 10^{-4} M) caused a significant increase in protein, IGF-I, or TGF-β1 concentrations in the culture medium. The expression of IGF-I or TGF-β1 mRNAs were significantly increased after

culture with zinc (10^{-4} M). Zinc has been shown to have stimulatory effects on IGF-I or TGF-β1 production in the femoral tissues with bone growth of newborn rats [62].

Bone growth factors are physiologically important in the stimulation of bone growth of newborn rats [63, 64]. Zinc has a physiologic role in the stimulation of bone growth in collaboration with IGF-I or TGF-β1 [65]. Culture with zinc has been shown to enhance the effect of IGF-I in increasing the culture medium protein concentration, alkaline phosphatase activity, or DNA content in the femoral-diaphyseal and -metaphyseal tissues obtained from newborn rats. Such an effect of zinc was not seen in the case of TGF-β1. Zinc may partly act on protein tyrosine kinase and protein tyrosine phosphatase that are related to signaling mechanism of IGF-I in the cells [43].

Zinc has been shown to stimulate in vitro DNA synthesis activity in the femoral-diaphyseal and -metaphyseal tissues obtained from newborn rats [66]. An increase in bone DNA synthesis activity was associated with bone growth of newborn rats with aging (1–21 days). Bone DNA synthesis activity has been shown to reduce after culture with zinc chelator in the reaction mixture of assay system for DNA synthesis [66], indicating that bone endogenous zinc plays a role in the enhancement of bone DNA synthesis associated with bone growth of newborn rats. Also, zinc has a direct stimulatory effect on DNA synthesis in the femoral tissues obtained from newborn rats [66]. DNA polymerase, which is related to DNA synthesis, is a zinc enzyme. It is possible that zinc partly stimulates DNA synthesis due to activating DNA polymerase in osteoblastic cells of bone tissues.

Bone DNA synthesis activity has been significantly increased after culture with IGF-I or TGF-β1 [66]. The effect of IGF-I in increasing bone DNA synthesis activity was significantly enhanced in the presence of zinc. Such an effect was not seen in the case of TGF-β1. The effect of zinc, IGF-I, or zinc plus IGF-I in increasing bone DNA synthesis activity was completely prevented after culture with an inhibitor of mitogen-activated protein (MAPK) kinase. Zinc has a stimulatory effect on bone DNA synthesis in newborn rats. Zinc has been demonstrated to increase proliferation of osteoblastic MC3T3-E1 cells in vitro [39]. Presumably, zinc activates MAPK kinase, and it has a stimulatory effect on the proliferation of osteoblastic cells in the bone tissues of newborn rats [64]. Zinc deficiency has been reported to induce a decrease in serum IGF-I level and a retardation of skeletal growth in young rats [5]. Zinc may play an important role in the stimulation of bone growth in collaboration with IGF-I in newborn rats.

As mentioned above, the cellular and molecular mechanism by which zinc stimulates bone growth in newborn rats has been shown in Fig. 3.

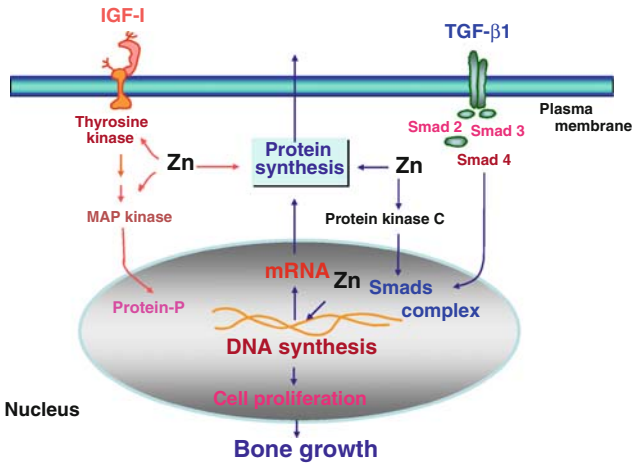

Fig. 3 Role of zinc in the stimulation of bone growth. Zinc increases protein synthesis at translational process due to activating aminoacyl-tRNA synthetase in osteoblastic cells. Zinc activates MAPK kinase or protein kinase C that is related to signaling in gene expression, and it may directly enhance gene expression. Zinc also stimulates DNA synthesis in the cells. Moreover, zinc increases production of IGF-I and TGF-β1 which act osteoblastic cells, and the metal modulates the stimulatory effects of IGF-I and TGF-β1 on cell proliferation and their related cell functions in the cells

Zinc stimulates fracture healing

Fracture repair can be considered as a biologically optimal process resulting in the restoration of injured skeletal tissue to a state of normal structure and function [67]. The mechanism by which bone fracture heals is complex. Fracture healing can be envisioned as involving five distinguishable processes, including the immediate response to injury, intramembranous bone formation, chondrogenesis, endochondral bone formation leading to the reestablishment of load bearing function, and bone remodeling [67, 68]. It is recognized that these processes may occur simultaneously during fracture repair. During fracture healing, a number of growth factors, cytokines, and their cognate receptors are present at elevated levels in and around the fracture site [69].

Zinc has been shown to stimulate fracture healing. The role of zinc in fracture healing has been determined using the diaphyseal tissues obtained at 7 or 14 days after the fracture of femoral diaphysis of rats [68–71]. Protein content in the femoral-diaphyseal tissues was markedly increased in fracture healing, and many protein molecules were produced in the bone tissues with healing [70]. When the diaphyseal tissues with fracture healing were cultured, the significant increase in bone alkaline phosphatase activity and DNA content is caused. These increases were significantly enhanced after culture with zinc compound [70]. These increases were completely prevented after culture with an inhibitor of protein synthesis.

Culture with diaphyseal tissues of fracture healing caused a significant increase in IGF-I or TGF-β1 in culture medium [71]. The production of IGF-I or TGF-β1 from bone tissues with fracture healing was significantly enhanced after culture with zinc compound. The effect of IGF-I or TGF-β1 in increasing protein content in the bone tissues with fracture healing was enhanced after culture with zinc compound [71].

When the femoral-diaphyseal tissues obtained from rats with fracture healing were cultured for 24 h in a serum-free medium, many proteins in the bone tissues were released into the culture medium [72]. Especially, a protein molecule of approximately 66 kDa was markedly increased with fracture healing. The increase in 66 kDa molecule was enhanced after culture with zinc compound [72]. The effect of zinc in increasing the 66 kDa molecule was based on a newly synthesized protein. Production of bone osteocalcin, which is significantly increased during fracture healing, has been shown to enhance after culture with zinc compound [73].

The results of N-terminal sequencing of 66 kDa protein showed that the N-terminus is identical to that of rat albumin [74]. The expression of albumin was seen in the diaphyseal (cortical bone) and metaphyseal (trabecular bone) tissues of rat femur. Albumin production in the bone tissues with fracture healing has been shown to increase after culture with PTH, IGF-I, or zinc compound [74]. Albumin has been demonstrated to express in bone marrow cells and osteoblastic MC3T3-E1 cells [75]. Albumin has been shown to stimulate the proliferation of osteoblastic MC3T3-E1 cells, and it suppresses alkaline phosphatase activity that is a marker enzyme of the differentiation of the cells in vitro [76]. Albumin has also been shown to have a suppressive effect on Runx2 (type 1) mRNA expression and a stimulatory effect on α1 (I) collagen mRNA level in osteoblastic MC3T3-E1 cells in vitro [77]. Albumin may play a physiologic role in bone formation and mineralization in osteoblastic cells.

Thus, fracture healing induces a remarkable production of albumin, which has an anabolic effect on bone, in the femoral-diaphyseal tissues of rats. Zinc promotes fracture healing due to increasing many bone protein components including albumin, IGF-I, and TGF-β1 which can stimulate osteoblastic bone formation.

Bone fracture often occurs in osteoporosis. Chemical factors that can stimulate the healing of bone fracture have not been fully developed. The daily oral administration of zinc acexamate (100 mg Zn/kg) for 28 days caused a significant increase in calcium content, alkaline and acid phosphatase activities, protein and DNA contents in the femoral-diaphyseal tissues of rats with fracture healing [78]. The supplemental intake of zinc compound has a usefulness in the promoting of fracture healing of the femoral-diaphyseal tissues in rats.

Preventive effect of zinc compound on bone loss

β-Alanyl-L-histidinato zinc (II) (AHZ), in which zinc is chelated to β-alanyl-L-histidine, is a new zinc compound. Its molecular weight is 289.61. The oral administration of AHZ has been found to stimulate bone growth in weanling rats, and the compound has a potent effect in comparison with zinc sulfate [79]. AHZ has been demonstrated to stimulate bone formation in tissue culture [80] and proliferation and differentiation of osteoblastic cells in vitro [39, 40]. These effects of AHZ were potentially in comparison with that of zinc sulfate.

AHZ is easily absorbed from the intestine. The zinc in AHZ may largely accumulation in bone cells without difficulty because the metal binds to the hydroxyapatite of bone tissue [81]. AHZ can easily enter bone cells (osteoblasts and osteoclasts) in comparison with zinc sulfate [82]. This explains the different effects of AHZ and zinc sulfate on the cell functions.

AHZ has a potent stimulatory effect on protein synthesis in osteoblastic cells [83]. Aminoacyl-tRNA synthetase is an enzyme that synthesizes aminoacyl-tRNA, and the enzyme in osteoblastic cells was directly activated by AHZ (10^{-7} to 10^{-5} M). The effect of AHZ in increasing aminoacyl-tRNA synthetase activity was greater than that of zinc sulfate [84]. AHZ may be able to bind easily to aminoacyl-tRNA synthetase. β-Alanyl-L-histidine does not have an effect. Zinc ion is required in the revelation of AHZ action, since the action is completely abolished in the presence of dipicolinate, a chelator of zinc ion.

The effect of zinc sulfate, AHZ, di(*N*-acetyl-β-alanyl-L-histidinato) zinc, and di(histidino) zinc on bone metabolism is also compared in vivo and in vitro [83, 84]. The chemical form of zinc-chelating β-alanyl-L-histidine has been demonstrated to reveal a potent-anabolic effect on bone formation and calcification in some dipeptides used as ligand.

AHZ has been shown to have a preventive effect on bone loss with various pathophysiologic conditions.

Zinc content in the cellular components, but not the matrix, has been demonstrated to be lower in the femoral diaphysis of aged rats (30 weeks old) than in that of weanling rats (3 weeks old) [85, 86]. Bone protein synthesis most likely deteriorates with increasing age. This deterioration has been found to restore after the oral administration of zinc (5–20 mg Zn/kg) for 3 days. The depletion of zinc in bone cells may cause the deterioration of bone formation in aged rats. The decrease in bone zinc with aging may play a role in the development of bone loss with increasing age. The supplementation of zinc may be

important in the prevention of bone loss with aging. AHZ (10–75 mg/kg body weight/day) was orally administered to aged rats (30 weeks old). The administration caused a significant increase in zinc, calcium, and DNA contents in the femoral diaphysis [87]. The anabolic effect of AHZ has also been shown in the tissue culture using bone obtained from aged rats in vitro [88].

It is known that skeletal unloading caused by immobilization, spaceflight, bed rest, or hindlimb suspension results in osteopenia. Zinc content has been shown to be decreased in the femoral-metaphyseal tissues of rats with skeletal unloading [89, 90]. Skeletal unloading results in an inhibition of bone formation and induces an increase in bone resorption, and thereby decreasing in bone mass [91]. Skeletal unloading was designed using the model of hindlimb suspension in rats that we developed. Animals were fed for 4 days with the unloading. The unloading induced a significant decrease in metaphyseal zinc content. Zinc accumulation into the metaphyseal tissues after a single oral administration of zinc sulfate (200 mg Zn/kg) was depressed after the unloading [89]. Serum zinc concentration in skeletal-unloaded rats was higher than that in normal rats. The impaired movement of zinc from serum into bone tissues may be caused with the unloading [89]. Skeletal unloading caused a significant decrease in bone components [89, 90]. Skeletal unloading-induced decrease in zinc content, alkaline phosphatase activity, and DNA content in the femoral diaphysis of rats was restored after the oral administration of AHZ (25–100 mg/kg/day) [92]. The effect of zinc has been shown to involve in newly synthesized proteins. The supplementation of zinc compound may have a role in the prevention of bone loss with skeletal unloading.

Aluminum has been shown to localize in the bone from patients with renal failure, and this induces bone disorders [93]. The dose of 2.7 and 5.4 mg Al/kg caused a significant increase in serum calcium concentration and bone acid phosphatase activity, while bone alkaline phosphatase activity and calcium content were not significantly altered [94]. The bone DNA content was significantly decreased with the dose of 5.4 mg Al/kg. These decreases of bone components were completely blocked after the simultaneous administration of AHZ (10 and 25 mg/kg). AHZ can prevent the revelation of the toxic effect of aluminum on bone metabolism in rats [94].

Feeding with low-calcium and vitamin D-deficient diets induces a decrease in serum calcium concentration and a corresponding fall in bone calcium content. When rats were fed on low-calcium (0.10%) and vitamin D-deficient diets for 14 days, the decrease in serum calcium and bone calcium was induced [95]. When AHZ (10–100 mg/kg/day) was orally administered to rats fed low-calcium and vitamin D-deficient diets for 14 days, the administration resulted in an increase in bone components [95]. The serum calcium and inorganic phosphorus concentrations were not significantly altered after AHZ administration. AHZ may directly stimulate bone formation independently of alteration in serum mineral homeostasis.

The inhibition of bone formation occurs during acute inflammation in the rat, and changes in osteoblast function are part of the acute phase response following local inflammation. Bone loss was seen in rats with adjuvant arthritis; local subcutaneous injection of 1% *Mycobacterium butyricum* suspension was used to induce systemic inflammatory response in rats. The decrease in calcium content and alkaline phosphatase activity were seen in the femoral diaphysis of rats with adjuvant arthritis [96]. The oral administration of AHZ (30 and 100 mg/kg) produced a significant increase in alkaline phosphatase activity, DNA, calcium, and zinc contents in the femoral tissues of rats with adjuvant arthritis. AHZ did not have a direct inhibitory effect on inflammation. AHZ (300 mg/day) treatment has been shown to improve periarticular osteoporosis, probably through an increase of bone formation, in postmenopausal women with rheumatoid arthritis [97].

Glucocorticoid therapy induces the development of secondary hyperparathyroidism and the inhibition of osteoblastic function [98]. The steroid treatment caused a significant increase in serum PTH level, while serum calcium, inorganic phosphorus, and zinc concentrations were not significantly altered [99]. The femoral-diaphyseal alkaline phosphatase activity, DNA, and calcium contents were significantly decreased after the administration of steroid, although bone zinc content was not significantly changed. When AHZ (30 or 100 mg/kg) was orally administered for 30 days to rats administered with the steroid, the administration completely prevented the increase in serum PTH level and the decrease in the bone components caused by the steroid treatment. Bone zinc content was significantly increased after the administration of AHZ. AHZ may have a therapeutic effect in the steroid-induced osteoporosis.

Ovariectomy causes a lack of estrogen. Estrogen deficiency induces osteoporosis in humans and in rats [100, 101]. Ovariectomy caused a significant decrease in alkaline phosphatase activity, DNA, and calcium contents in the femoral diaphysis of rats [102–104]. These decreases were completely prevented after the oral administration of AHZ (10–100 mg/kg/day) for 6 weeks [102]. AHZ restored bone loss in ovariectomized rats. Moreover, rats were fed for 3 weeks after ovariectomy, and then the animals were orally administered AHZ (10–100 mg/kg/day) for 3 months [103, 104]. A remarkable decrease in estrogen concentration in rat serum was seen 3 months after ovariectomy. The prolonged oral administration of AHZ did not influence serum estrogen level after ovariectomy.

The administration of AHZ completely prevented reduction of bone mass [103]. AHZ administration for 3 months could completely prevent reduction in mineral content in the trabecular and cortical bone tissues of ovariectomized rats [104]. AHZ has been shown to have a preventive effect on ovariectomy-induced bone loss.

Zinc acexamate has been shown to have a potent effect on bone formation as compared with that of zinc sulfate and AHZ in vitro [105]. Zinc acexamate may be a good tool in therapy of osteoporosis. Bone loss has been shown to induce with diabetes [106, 107]. Streptozotocin (STZ)-diabetic rats induce an impairment of insulin secretion. A single subcutaneous administration of STZ (60 mg/kg body weight) to rats caused a significant decrease in body weight and serum zinc concentration, the increase in serum glucose and triglyceride levels, and the reduction of alkaline phosphatase activity, calcium, and DNA contents in the femoral-diaphyseal and -metaphyseal tissues of STZ-diabetic rats [108, 109]. Zinc acexamate (25 mg Zn/kg) was orally administered once daily for 14 or 21 days to rats received a single subcutaneous administration of STZ. Zinc acexamate had potent-preventive effects on the changes in body weight, serum findings, and bone loss in STZ-administered rats, indicating its restorative effect on insulin-dependent (type I) diabetic conditions [107, 109]. Zinc acexamate had a potent-preventive effect on diabetic conditions as compared with zinc sulfate. Zinc acexamate may have preventive and therapeutic effects on diabetes-induced bone loss in rats.

Nutritional zinc and osteoporosis

Osteoporotic patients have been shown to have lower levels of skeletal zinc than healthy individuals [19]. The reduction levels of biological markers of nutrition in postmenopausal osteoporosis may be related to zinc deficiency. In postmenopausal women, urinary zinc has been used as a marker of bone resorption. Plasma and urinary zinc concentrations in 30 women with postmenopausal osteoporosis and in 30 healthy postmenopausal women who served as controls have been measured [110]. Plasma zinc levels did not differ between groups, but urinary zinc excretion has been found to be significantly higher in the women with postmenopausal osteoporosis [110]. The elevation of urinary zinc elimination in osteoporosis may be dependent on bone resorption [110] because zinc is located richly in bone tissues. Measurement of urinary zinc may be a useful biochemical method of observing the positive clinical effect following alendronate or calcitonin therapy in postmenopausal women [111].

The relationship between indices of zinc nutritive status and biochemical markers of bone turnover in older adult European subjects has been shown [112]. A total of 387 healthy adults, aged 55–87 years was used in this study. There was seen some, albeit inconsistent, evidence of a relationship between zinc nutritive status and bone turnover in the older adult participants of the ZENITH study [112].

The supplements of trace minerals with or without calcium in postmenopausal women have been shown to have beneficial effects on bone density [20]. Low zinc intakes and reduced blood zinc concentrations have been reported to be associated with osteoporosis in women [113]. To examine the independent association between dietary zinc and plasma zinc and the association of each with bone mineral density (BMD) and 4-year bone loss in community-dwelling older men. Of the original Rancho Bernardo Study subjects, 396 men (age: 45–92 years) were used. The mean dietary zinc intake was 11.2 mg. Dietary zinc intake and plasma zinc each have a positive association with BMD in men [113].

Protein undernutrition is frequent in the elderly. It contributes to the development of osteoporosis, possibly via lower IGF-I. Dietary zinc can influence IGF-I production. In the elderly, zinc supplementation accelerated the serum IGF-I response to essential amino acids–whey protein supplements by 1 week and decreased a biochemical marker of bone resorption [114].

Changes in circulating biochemical markers of bone metabolism in aged individuals with the food intake supplemented with zinc has been examined [115]. Sixty-three volunteers (31 men and 32 women) were divided into four groups of 15 or 16 male volunteers and 16 or 16 female volunteers, and each group was sequentially food containing 0.8 or 3.6 mg zinc once a day for 4 or 8 weeks as follows. The dietary intake of zinc for 8 weeks in men or women caused a significant increase in serum bone-specific alkaline phosphatase activity and γ-carboxylated osteocalcin concentration and a significant decrease in serum bone TRACP activity and N-telopeptide of type I collagen, as compared with the control group without dietary zinc [115]. This study suggests that the supplementation with zinc has a stimulatory effect on bone formation and an inhibitory effect on bone resorption in aged individuals.

Intake of dietary zinc may have a benefit effect in the prevention of osteoporosis.

Prospect

Nutritional zinc is abundant in bone and may act as a local regulator of bone cells. Zinc plays a physiologic role in the regulation of bone homeostasis. Zinc stimulates osteoblastic bone formation and mineralization, and it inhibits

osteoclastic bone resorption, thereby increasing bone mass [116–118]. Zinc transporter has been shown to locate in osteoblastic [119] and osteoclastic cells [120]. Zinc transporter may be important role in the uptake, intracellular sequestration, or efflux of zinc. Intracellular zinc stimulates the synthesis of many proteins at translational process due to activating aminoacyl-tRNA synthetase and to stimulate gene expressions in osteoblastic cells. Zinc also increases DNA synthesis in the cells. The molecular mechanism of zinc on nuclear function in osteoblastic cells remains to be elucidated. In addition, the regulatory mechanism of intracellular zinc in osteoclastic cells has been poorly understood, although zinc stimulates apoptosis of osteoclastic cells which are mediated through Ca^{2+} signaling.

There are growing evidences that zinc finger transcription factors as zinc-binding protein play an important role in differentiation of osteoblastic and osteoclastic cells. A novel zinc finger-containing transcription factor, called Osterix (Osx), has been found in osteoblastic cells [121]. Osx is specifically expressed in all developing bones. Osx is required for osteoblast differentiation and bone formation, and it acts downstream of Runx2/Cbfa1. Cas-interacting zinc finger protein (CIZ) has been found to be a novel type inhibitor of bone morphogenetic protein (BMP)/Smad signaling in the modulation of BMP2-induced osteoblastic cell differentiation [122]. Moreover, Schnurri-3 (Shn3), a mammalian homolog of the Drosophila zinc finger adapter protein Shn, is an essential regulator of adult bone formation [123]. Runx2-mediated extracellular matrix mineralization is antagonized, revealing an essential role for Shn3 as a central regulator of postnatal bone mass. A novel TIZ (TRAF6-inhibitory zinc finger protein) has been shown to inhibit osteoclastogenesis and the function of TNF receptor-associated factor 6 [124]. TIZ may play a regulatory role during osteoclast differentiation by modulating TRAF6 signaling activity. Whether nutritional zinc state may affect on function of zinc finger proteins has been poorly understood. Presumably, zinc deficiency attenuates function of these proteins.

The decrease in bone zinc with pathophysiologic state has been shown to lead bone loss. The supplemental intake of zinc prevents bone loss. Zinc yeast has been shown to have a high bioavailability in rats and its dietary intake has an anabolic effect on bone components in vivo [125], indicating its usefulness as a functional food ingredient.

β-Alanyl-L-histidinato zinc (AHZ) or zinc acexamate has been shown to have a potent-anabolic effect in comparison with zinc sulfate. These compounds may be a clinical usefulness in the therapy of osteoporosis.

Thus, zinc supplementation may play an important role in the prevention and therapy of osteoporosis. Further development is expected.

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