Nutritional Sciences 8(4), November, 2005, pp.242-249

Zinc Deficiency Decreased Alkaline Phosphatase Expression and Bone Matrix Ca Deposits in Osteoblast-like MC3T3-E1 Cells*

Young-Eun Cho, Ria-Ann R. Lomeda, Yang-Ha Kim[1], Sang-Hoon Ryu[2], Je-Yong Choi[3], Hyo-Jin Kim[3], John H. Beattie[4], In-Sook Kwun§

Department of Food Science and Nutrition, Andong National University, Kyungpook 760-749, Korea,
[1]Department of Food Science and Nutrition, Ewha Womens University, Seoul 120-750, Korea,
[2]Department of Central laboratory for Instrumental Analysis, Andong National University, Kyungpook 760-749, Korea,
[3]Department of Biochemistry, School of Medicine, Kyungpook National University, Daegu 701-702, Korea,
[4]Cellular Integrity Division, Rowett Research Institute, Aberdeen, Scotland, United Kingdom

It is well established that zinc plays an important role in bone metabolism and mineralization. The role of zinc in bone formation is well documented in animal models, but not much reported in cell models. In the present study, we evaluated zinc deficiency effects on osteoblastic cell proliferation, alkaline phosphatase activity and expression, and extracellular matrix bone nodule formation and bone-related gene expression in osteoblastic MC3T3-E1 cells. To deplete cellular zinc, chelexed-FBS and interpermeable zinc chelator TPEN were used. MC3T3-E1 cells were cultured in zinc concentration-dependent (0-15 μM $ZnCl_2$) and time-dependent (0-20 days) manners. MC3T3-E1 cell proliferation by MTT assay was increased as medium zinc level increased ($p<0.05$). Cellular Ca level and alkaline phosphatase activity were increased as medium zinc level increased ($p<0.05$). Alkaline phosphatase expression, a marker of commitment to the osteoblast lineage, measured by alkaline phosphatase staining was increased as medium zinc level increased. Extracellular calcium deposits measured by von Kossa staining for nodule formation also appeared higher in Zn+ (15 μM $ZnCl_2$) than in Zn- (0 μM $ZnCl_2$). Bone formation marker genes, alkaline phosphatase and osteocalcin, were also expressed higher in Zn+ than in Zn-. The current work supports the beneficial effect of zinc on bone mineralization and bone-related gene expression. The results also promote further study as to the molecular mechanism of zinc deficiency for bone formation and thus facilitate to design preventive strategies for zinc-deficient bone diseases.

Key words: Zinc deficiency, MC3T3-E1 cells, Alkaline phosphatase, Extracellular matrix Ca deposit, Osteocalcin

Received October 15, 2005; Revised November 7, 2005; Accepted November 18, 2005

INTRODUCTION

The human body contains 1-2 g of zinc (Zn) and about 90% is found in bone, muscle, skin and hair, while blood contains less than 1%. Zinc plays an important role in the regulation of bone formation and resorption and is essential for bone mineralization and osteoblast function.[1] Zinc affects the connective tissue metabolism, acting as a cofactor for several enzymes involved in the synthesis of various bone matrix constituents, such as alkaline phosphatase and collagenase. Alkaline phosphatase is necessary for bone mineralization and collagenase is essential for the development of the collagenous structure of bone.[2]

Zinc is required for osteoblastic activity, directly activating aminoacyl-tRNA synthetase in osteoblastic cells and stimulating cellular protein synthesis. In addition, zinc increases bone mineralization through its role as a cofactor for alkaline phosphatase and protein tyrosine phosphatase.[3] Bone growth retardation is a common finding in Zn deficiency, both with experimentally- induced deficiency in growing animals and in children as a result of dietary insufficiency. It was reported that Zn concentration in the bone of patients with osteoporosis was reduced.[4] Also, studies in rats have shown that zinc deficiency results in a reduction in femur zinc concentration,[5] a reduction in cancellous bone mass and a deterioration of trabecular bone architecture.[6] The role of zinc in bone is two fold: firstly, zinc plays a structural role in the bone matrix,

* This research was supported by Korea Science and Engineering Foundation (KOSEF)/Korea Research Foundation grant, No. R05-2004-000-11036-0.
§ To whom correspondence should be addressed.
(E-mail : iskwun@andong.ac.kr)

since bone mineral is composed of hydroxyapatite crystals which contain zinc complexed with fluoride. Secondly, zinc is involved in the stimulation of bone formation by osteoblasts and the inhibition of bone resorption by osteoclasts.[5] Bone is composed of several collagenous (pro-or collagen type 1) and non- collagenous (osteocalcin, osteopontin, osteonectin, etc.) proteins in the extracellular matrix. All of these can be markers of bone formation, as well as alkaline phosphatase (ALP) which affects matrix- vesicle-mediated mineralization. Alkaline phosphatase is a key enzyme for the formation and calcification of the bone tissues. The preosteoblasts differentiate into osteoblasts and then produce alkaline phosphatase, and process procollagens to collagens, and finally the cells form mineralized tissues *in vitro*. Alkaline phosphatases of MC3T3-E1 cells were released into the medium during cell culture, and the time course of the increase of alkaline phosphatase in the medium was consistent with the progress of mineralization[7]. Thus, it can be considered that matured and released ALP from the cells might be closely related to the mineralization. Alkaline phosphatase is also known as zinc-dependent enzyme.[2]

In spite that zinc is essential for bone mineralization and osteoblast function, only limited studies for zinc effects on osteoblasts have been reported. In the present study, we determined whether zinc deficiency 1) would affect cell proliferation and viability, 2) would affect alkaline phosphatase activity and expression, and 3) particularly affect extracellular matrix bone nodule formation and bone-related genes expression in osteoblastic MC3T3-E1 cells.

MATERIALS AND METHODS

1. Cell Culture and Zn-treatment Using Zn-chelexed FBS and TPEN/$ZnCl_2$

Chelex-treated fetal bovine serum (Sigma) was used to limit the zinc availability in composed media. Chelex-100 ion exchange resin (5 g: Bio-Rad) was added to fetal bovine serum (FBS, 100 mL), and the mixture was stirred overnight at 4 ℃. After removal of the resin, the solution was then filter-sterilized into polyethylene centrifuge tubes. Zn depleted media was then prepared using α-minimum essential medium (α-MEM: Gibco) supplemented with 10% fetal bovine serum (Gibco) and 100 units/mL penicillin and 100 g/mL streptomycin (Gibco), 100 mL/L chelexed FBS.[8,9] All plasticwares used for media preparation and cell culture work were washed with Acationox detergent (Baxter Scientific Products, McGaw Park, IL) before use.

Preosteoblast cell line, MC3T3-E1 (ATCC, CRL-2593) were seeded at a density of 3×104 cells/cm^2 (100 mm culture dish, Corning, PA, USA) and cultured in regular growth culture media consisting of α-minimum essential medium (α-MEM: Gibco) supplemented with 10% fetal bovine serum (Gibco) and 100 units/mL penicillin and 100 g/mL streptomycin (Gibco) in a humidified atmosphere of 5% CO_2 at 37℃: At 70~80% confluence, the cells were cultured in chelexed-FBS containing osteogenic media [regular media described above plus 10 mM β -glycero-phosphate (Sigma) and 50 g/mL L-ascorbic acid (Sigma)] for 30 days with various zinc levels (1~15 μM) as $ZnCl_2$ and 5 μM *N,N,N',N'*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) as zinc chelator. An intracellular zinc chelator TPEN *N,N,N',N'*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) was made as 100 mM/1 mM (200-fold) stock solution in deionized water and diluted to working concentrations at the time of experiments. Chelation of intracellular zinc with the cell-permeant chelator TPEN (5 μM) was achieved for appropriate treatment periods. In experiments where needed, indicated factors were directly added (usually at 100-fold dilution from stock solutions) to the medium at desired concentrations. Culture media were changed every 2~3 days and the cells were harvested every 5 days up to 30 days. Osteoblastic MC3T3-E1 cells showed the mature bone nodules and cell morphology about up to 30 days. All reagents for cell culture and zinc/TPEN treatment were products of Gibco and Sigma. The media containing chelex-treated FBS was used for Zn-deficiency treatment. For the Zn-adequacy treatment, the media containing chelex- treated FBS was supplemented with a $ZnCl_2$ to a nominal level of 0~15 μM Zn.

2. Cell Viability and Proliferation : Microscopic and MTT Assay

MC3T3-E1 cell death was morphologically (microphotographs or phase-contrast photomicrographs) assessed under the microscope after TPEN treatment for each appropriate treatment periods up to 30 days. The viability of MC3T3-E1 was determined by MTT for every other 5-day period during the whole 30 days. Staining for 3-[4,5-dimethylthiazol -2-y]-2,5-diphenyltetrazolium bromide (MTT) reduction, an indirect indicator of cell viability, was done. In some experiments, trypan blue exclusion tests were performed. Cultures were exposed to 0.4% trypan blue for 5 min, and then washed three times with α-MEM. Cultures were observed and photographed under the bright field microscope.

3. Extra- and Intra-Cellular Zn and Bone-related Minerals (Ca, P) and Protein Assay

Cells were wet-digested using trace element free concentrated nitric acid (Fluka, Switzerland). The wet-digested samples were diluted with trace element free 0.125 M HCl (Fluka, Switzerland). The diluted samples were filtered using 0.45 m syringe filters (Corning, New York, USA) and measured using inductively coupled plasma emission spectroscopy (BoschstraBe 10, Spetro Analytical Instruments, Germany) for zinc and bone-related minerals (Ca, P). The analytical accuracy of the method for mineral analysis was tested using a standard reference material (SRM) obtained from the National Institute of Standards and Technology (NIST SRM 1577b, bovine liver, USA). The certified Zn and Ca values of SRM were 127±16 μg/g and 160±8 μg/g, respectively and the measured Zn and Ca values were 120±7 μg/g and 154±7 μg/g, which corresponded to 94.5% and 96.0% of the reference values for Zn and Ca, respectively (n = 2). Cellular protein concentration was analyzed by Lowry method[11] with bovine serum albumin as the standard.

4. Cellular Alkaline Phosphatase (ALP) Activity Assay

Cellular (anchored) alkaline phosphatase was measured by enzymatic activities. Cells were washed with PBS and lysed in 1 mL of 0.02% Nonident P-40 (Sigma). The lysates were sonicated for 30 s twice on ice. The sonicated lysates were centrifuged for 15 min at 12,000 g. The supernatant was kept at -20 ℃ until analysis. The activity of ALP in lysates was measured by using p-nitrophenyl phosphate as the substrate and the optical density of 405 or 410 nm was determined as previously described.[10] Protein concentration was estimated by the method of Lowry *et al.*,[11] with bovine serum albumin as the standard. The activity of ALP was expressed as mU/mg of protein.

5. Alkaline Phosphatase Staining

MC3T3-E1 cells grown in α-MEM supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and antibiotics were treated with various zinc levels (0, 5, 10, 15 μM Zn as $ZnCl_2$) and intracellular zinc chelator (5 μM TPEN), which was used to deplete cellular zinc levels, up to 20 days. MC3T3-E1 cells used as controls were grown under the same condition using the normal differentiation medium without the addition of TPEN. Staining for alkaline phosphatase was performed on treated and untreated control cells, 10 and 20 days after zinc treatment. The cells were rinsed with phosphate buffered saline (PBS) and stained using the mixture of Napthol As-Mx phosphate disodium salt, N,N-dimethyl formamide and fast red salt (all reagents from Sigma) for 30 min at 37 ℃, or until yellow in appearance. After washing with distilled/ deionized water, the cells were photographed.

6. von Kossa Staining and Mineralized Nodules Formation

Since calcium usually co-precipitate with phosphate ions *in vitro* culture condition, mineralization of the nodules in the cultures was assessed using von-Kossa stain. The matrix was washed with PBS, and cultures were treated with 5% silver nitrate solution 100 mL/well in the dark at 37 ℃ for 30 min. The excess silver nitrate solution was then completely washed away using distilled/ deionized H_2O and the culture plate was exposed to sodium carbonate/ formaldehyde solution for few minutes to develop color. Mineralized and unmineralized nodules could be distinguished separately: mineralized nodules by their von-Kossa-positive staining (dark brown center and light brown peripheral area), and unmineralized nodules by their surface layer of cuboidal cells, light brown staining, and three-dimensional structure. To evaluate bone nodule area precisely, the von-Kossa-stained areas were viewed through light microscopy.

7. RT-PCR for Bone-related Gene Expression

The guanidinium-thiocyanate method using Trizol Reagent (Gibco BRL, USA) was used for adipose tissue RNA extraction.[12] For first-strand cDNA synthesis, 100 ng of RNA from each sample was reverse transcribed using 20 U of AMV reverse transcriptase and Oligo-p $(dT)_{15}$ 1X random primers (Roche Diagnostics, USA). The resulting cDNAs were PCR-amplified by using a mixture of the corresponding sense and antisense primers. Primers for target and housekeeping genes are shown in Table 1. The PCR conditions were 95 ℃ for 10 min and then 35 cycles at 95 ℃ for 1 min, 60 ℃ for 1 min and 72 ℃ for 1 min and a final extension at 72 ℃ for 5 min. The PCR products were separated on 1.2 % agarose gel.

Table 1. Primer base sequences for target and housekeeping genes

Genes		Base sequences
Alkaline phosphatase	sense	5' GCT GAT CAT TCC CAC GTT TT 3'
	antisense	5' CTG GGC CTG GTA GTT GTT GT 3'
Osteocalcin	sense	5' AAG CAG GAG GGC AAT AAG GT 3'
	antisense	5' TTT GTA GGC GGT CTT CAA GC 3'
Osteopontin	sense	5' TGC ACC CAG ATC CTA TAG CC 3'
	antisense	5' CTC CAT CGT CAT CAT CAT CG 3'
PTH receptor	sense	5' GGG CAC AAG AAG TGG ATC AT 3'
	antisense	5' GGC CAT GAA GAC GGT GTA GT 3'

8. Statistical Analysis

Data were analyzed with SPSS program and differences were considered significant at $p<0.05$. Statistical analysis of the data was performed by one-way ANOVA to test the effect of the different Zn levels. Once significance was detected, Tukey's HSD test was used for comparison of difference between groups.

RESULTS

1. Zn Level in Medium Components Using Chelexed FBS

The Zn concentration of various media using normal and chelexed FBS is shown in Table 2. The Zn levels in Zn adequate and deficient media were nominally 0-15.0 μM Zn. Most of the trace elements were contained in the serum source, and the medium itself contained very little amount of trace element under culture condition. The Zinc concentration of Zn depleted medium (α-MEM +chelexed FBS) (0.651 μM) was about one fourth level of the normal growth medium (α-MEM+FBS) (2.723 μM) zinc level as measured by atomic absorption spectroscopy. Cu and Fe concentrations in chelexed-FBS were not changed, thus chelexing only removed Zn in FBS. TPEN treatment showed the same pattern of chelexting treatment.

Table 2. Zn concentration in various cell medium components containing normal and chelexed-FBS for cellular zinc depletion[1)]

Media component	Zn concentration (g/mL)	Zn concentration (M)
MEM	0.021±0.009	0.326±0.059
FBS	2.537±0.160	39.023±4.160
Chelexed FBS	0.002±0.000	0.023±0.009
-MEM+FBS	0.177±0.030	2.723±0.530
-MEM+chelexed FBS	0.042±0.008	0.651±0.048
-MEM+chelexed FBS+15 M $ZnCl_2$	0.764±0.063	11.759±0.263

1) Mean±SD. Zn concentration was measured in duplicate.

2. Cell Morphology

The cells appeared to be morphologically unaffected by the Zn deficient treatment and were adherent to the plastic flasks and visually very similar to the Zn adequate cells up to 10~15 days. Thus, until harvesting of the cells up to 5 days, Zn-deficiency did not affect the cell's survival. After 15 days, the cells grew less as the zinc level decreased but did not show any apoptosis or necrosis status.

3. Effect of Zinc and Chelexed-FBS Medium on Cell Viability by MTT Assay

Since zinc is essential for cells, we determined how medium Zn level affected the osteoblastic-like cells. MC3T3-E1 cells were treated with different concentrations of zinc under chelexed-FBS containing medium for 15 days. Cell viability was assessed using the MTT assay. Increase of zinc concentration in the medium increased cell viability (Table 3). Cell viability showed less than 0.5 μM added medium Zn level decreased cell viability than the control medium. The results showed that zinc, in a dose-dependent manner within the range of 0.25~1 M $ZnCl_2$, increased cell viability, while <0.5 μM Zn level could induce cell death in this particular experimental conditions. The results imply a relationship among extracellular zinc or zinc depletion by chelexed-FBS and osteoblastic cell viability and confirm the positive effect of zinc on osteoblast viability.

Table 3. Cellular viability on MC3T3-E1 cells treated with various Zn levels for 15 days[1,2)]

Zn level	Cell viability (proliferation) % control
Control sister medium	100
Zn 0 μM	1.6±0.3^{d}
Zn 0.25 μM	29.6±1.4^{c}
Zn 0.5 μM	107.3±10.7^{b}
Zn 1 μM	164.2±18.6^{a}
Zn 3 μM	165.7±21.4^{a}
Zn 5 μM	171.9±24.1^{a}
Zn 15 μM	182.9±34.6^{a}

1) Mean±SD. Different superscript was statistically significant at $p<0.05$ by Tukey, ANOVA. Cellular viability was measured using MTT assay in triplicate.
2) Chelexed-FBS was used for cell medium preparation for the depletion of the medium and after then $ZnCl_2$ was added as for each appropriate zinc level.

4. Cellular Zn and Ca Levels

Cellular Zn and Ca concentrations under different medium Zn levels are shown in Table 4. Cellular Zn level was consistent among various medium Zn levels under this particular chelexed-FBS containing medium

Table 4. Cellular Zn and Ca concentration in MC3T3-E1 cells treated with various Zn levels[1,2)]

Medium Zn level	Zn level (g/mL)	Ca level (g/mL)
Zn 0 μM	0.029±0.008	0.093±0.014^{b}
Zn 3 μM	0.027±0.009	0.137±0.031^{b}
Zn 6 μM	0.027±0.009	0.102±0.023^{b}
Zn 9 μM	0.028±0.010	0.154±0.024^{b}
Zn 12 μM	0.032±0.009	0.171±0.031^{b}
Zn 12 μM	0.026±0.010	0.273±0.041^{a}

1) Mean±SD. Different superscript was statistically significant at $p<0.05$ by Tukey HSD, ANOVA. Zn and Ca were measured in triplicate.
2) Chelexed-FBS was used for cell medium preparation for depletion of the medium and after then $ZnCl_2$ was added for each appropriate zinc level. The cells were cultured at the various Zn levels for 20 days.

state. Zinc deficiency medium might cause translocation of zinc from extracellular to intracellular space where the cells can use Zn for basic cell activities. This would partly result from the need to maintain zinc and protect cells against any harmful effects due to zinc deficiency. Interestingly, cellular Ca level was increased as the medium Zn level increased (Table 4), even cellular Zn level was consistent among the various Zn levels. This means that Zn has a positive effect on the calcification of these MC3T3-E1 osteoblastic cells for bone formation.

5. Cellular Alkaline Phosphatase (ALP) Activity

We measured cellular ALP activity by various medium zinc levels in MC3T3-E1 cells. ALP, particularly bone-type ALP is supposed to play a key role in the formation and calcification of hard bone tissues. The levels of ALP in the cells are shown in Table 5. ALP activities of the cells were increased as the medium zinc level increased ($p<0.05$). ALP activity was greatly increased almost twice whenever medium zinc level was increased by 3 μM level. These results suggest that medium zinc level supports the calcification of osteoblastic-like cells through the supportive action to ALP formation.

Table 5. ALP activity in MC3T3-E1 cells treated with various Zn levels for 20 days[1,2)]

Zn level	ALP activity (mU/mg protein)
Zn 0 μM	0.026±0.001[b]
Zn 3 μM	0.045±0.007[b]
Zn 6 μM	0.042±0.004[b]
Zn 9 μM	0.071±0.005[a]
Zn 12 μM	0.157±0.007[a]
Zn 15 μM	0.355±0.263[a]

1) Mean±SD. Different superscript was statistically significant at $p<0.05$ by Waller-Duncan, ANOVA. ALP activity and protein concentration was measured in duplicate.
2) Chelexed-FBS was used for cell medium preparation for depletion of the medium and after then $ZnCl_2$ was added for each appropriate zinc level.

6. Alkaline Phosphatase Staining

In order to examine the effects of zinc on the maturation of MC3T3-E1 cells, we evaluated morphological changes and alkaline phosphatase expression. The cells were cultured for 10 and 20 days under different medium zinc levels and zinc chelator, TPEN (5 μM). The normal course of MC3T3-E1 development results in morphological changes from "fibroblastic" to "cuboidal" cell shape at this state. This transition did occur more as medium zinc level increased. Similarly, as shown in Fig. 1, the expression (red stains) of the alkaline phosphatase enzyme, a marker of commitment to the osteoblast lineage, was increased as medium zinc level increased. These results support

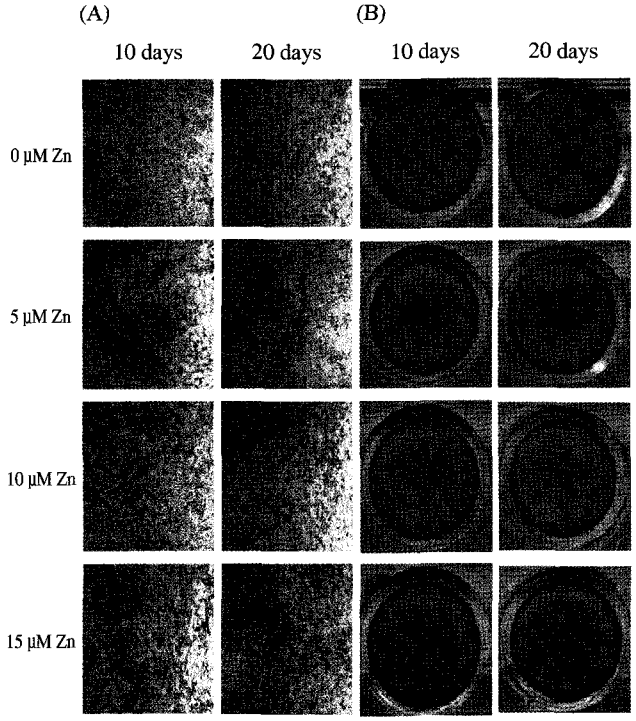

Fig. 1 Alkaline phosphatase staining on MC3T3-E1 cells treated with various Zn levels.

(A) Morphology of alkaline phosphatase staining on MC3T3-E1 cells treated with intracellular zinc chelator TPEN (5 M) and the various zinc levels as $ZnCl_2$.
(B) Alkaline phosphatase expression in MC3T3-E1 cells. Micrographs of MC3T3-E1 cells treated with the various zinc levels as $ZnCl_2$. Staining for alkaline phosphatase expression was performed on the cells 10 and 20 days after treatment.

that zinc affects ALP expression on bone formation in a positive way.

7. von Kossa Staining and Mineralized Nodules Formation

Calcium co-precipitates with phosphate ions *in vitro* culture condition, and von Kossa stain can bind with phosphate ions and thus can assess the mineralization of the nodules in the cultures. Mineralized and unmineralized nodules can be distinguished separately: mineralized nodules appeared to have a dark brown center and light brown peripheral area as shown in Fig. 2.

The cells were cultured for 5 days which was at the early stage of bone formation. Also, the results showed for both zinc depletion methods, chelexed-FBS and TPEN. Bone nodule formation was not prominent for 5 days of differentiation but Ca deposits were appeared as medium zinc level increased (Fig. 2). More nodules were found at higher zinc levels for both chelexed-FBS and TPEN treatments with appropriate zinc level addition. The results support that zinc affects collagenous bone matrix mineralization.

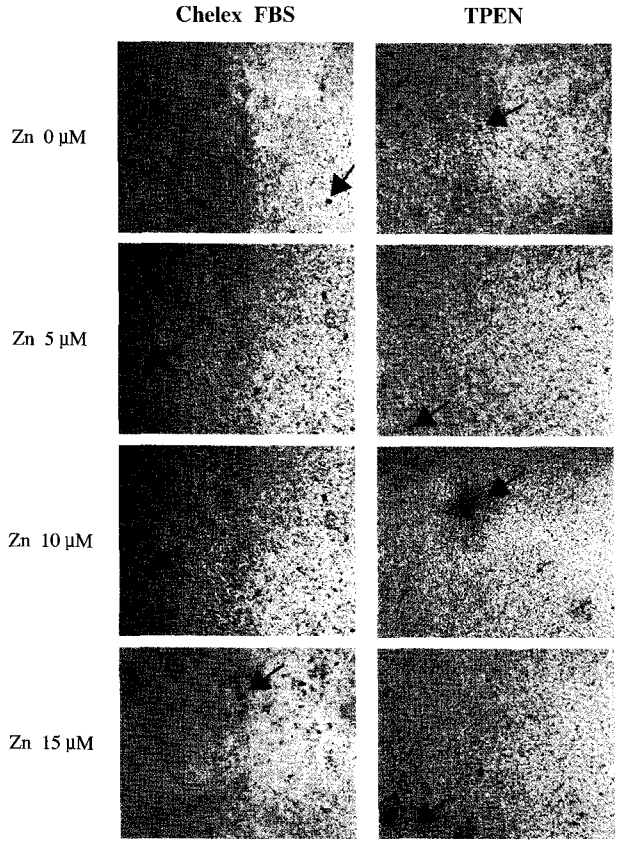

Fig. 2 von Kossa staining on MC3T3-E1 cells treated with various Zn levels for 5 days.

(A) Morphology of bone nodules on MC3T3-E1 cells treated with zinc chelator chelexed-FBS and the various zinc levels as $ZnCl_2$.

(B) Morphology of bone nodules on MC3T3-E1 cells treated with intracellular zinc chelator TPEN (5 μM) and the various zinc levels as $ZnCl_2$. Arrow points the nodule on extracellular matrix.

8. RT-PCR for Bone-related Gene Expression

The expression of extracellular matrix (ECM) mineralization positive (alkaline phosphatase, osteocalcin, osteopontin) and negative (parathyroid hormone receptor) bone marker genes in MC3T3-E1 cells was measured by RT-PCR. The cells were grown for 5 days, which was about the early stage of osteoblastic bone formation. Alkaline phosphatase was highly expressed in Zn+ (15 μM Zn) and osteolcalcin was highly expressed, but still less higher compared to alkaline phosphatase expression. Unexpectedly, osteopontin was expressed less in Zn+. Parathyroid hormone receptor was expressed evenly in Zn+ and Zn- at this early stage of bone formation.

Discussion

The role of zinc in bone formation is well documented in animal models,[12] but not much reported in cell models.

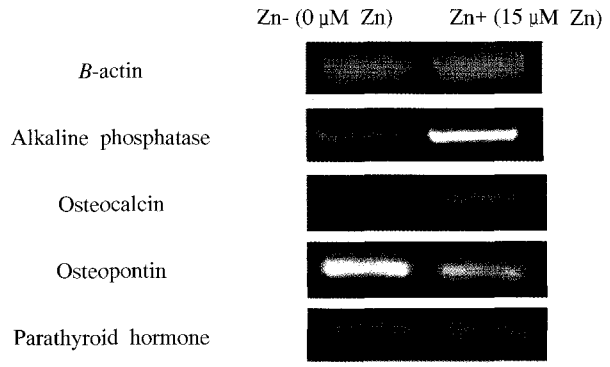

Fig. 3 Bone-related gene expression in MC3T3-E1 cells treated with various Zn levels for 5 days.

Cells were cultured in the medium which was composed with chelexed-FBS to deplete cellular zinc level and the various zinc levels as $ZnCl_2$. Bone-related gene mRNA levels quantified by RT-PCR. Total RNA from the cells was analyzed using specific primers and 18S ribosomal RNA was analyzed simultaneously as an internal control.

In animal model, zinc deficiency results in impaired DNA synthesis and protein metabolism, which lead to negative effects on bone formation,[2] and low serum levels of zinc are related to osteoporosis in humans.[13] In the present study, we evaluated effects of zinc on osteoblast cell proliferation, alkaline phosphatase activity and expression, and extracellular matrix calcification and bone-related genes expression in osteoblastic MC3T3-E1 cells.

The results of the present study demonstrate that zinc positively affects osteoblastic cell proliferation, alkaline phosphatase activity and expression, extracellular matrix bone nodule formation and selected bone-related gene expression in osteoblascitic MC3T3-E1 cells. MC3T3-E1 cells, a mouse clonal osteoblastic cell line, are known to form multiple layers and to accumulate mineralized extracellular matrices.[12] Generally, osteoblast development along osteogenic lineage consists of three stages: cell proliferation with extracellular matrix secretion, extracellular matrix maturation, and finally extracellular matrix mineralization[13] which is characterized by the expression of osteocalcin.

It has not been reported extensively that zinc can affect MC3T3-E1 cells' morphology, proliferation and differentiation. It can also be questioned that zinc status may induce MC3T3-E1 cells to switch from a proliferative state to a more differentiated state. In this study, we only evaluated the effects of zinc level (0~15 μM) to the viability of MC3T3-E1 cells and the results showed that zinc clearly affected cell viability (Table 3). Under the chelexed-FBS containing medium status, MC3T3-E1 cells inhibited viability upon<0.5 μM Zn addition into the medium in this experiment.

Alkaline phosphatase is mainly responsible for the formation and calcification of the bone tissues. The preosteoblastic MC3T3-E1 cells were differentiated into osteoblasts by adding vitamin C and glycerophosphate for the production of alkaline phosphatase.[16] The process was induced to convert procollagens to collagens and finally the cells form mineralized tissues *in vitro*. Also, alkaline phosphatases of MC3T3-E1 cells were released into the medium during cell culture, and the time course of the increase of alkaline phosphatase in the medium was consistent with the progress of mineralization.[11] Thus, it can be considered that matured and released alkaline phosphatase from the cells might be closely related to the mineralization. In the present study, we measured alkaline phosphatase activity, enzyme expression pattern on cell morphology using alkaline phosphatase staining, and also gene expression by RT-PCR. All of the data showed that zinc positively affected alkaline phosphatase activity and expression in osteoblastic MC3T3-E1 cells. As medium zinc level increased, cellular alkaline phosphatase activity was increased significantly ($p<0.05$) (Table 5). The results of alkaline phosphatase staining showed that this enzyme expression in cell multiple layers was zinc concentration- and time-dependent patterns (Fig. 1). Alkaline phosphatase gene was expressed prominently higher in Zn+ (15 μM $ZnCl_2$) than in Zn- (0 μM $ZnCl_2$). Zinc as zinc-containing organoapatite was increased in time-dependent manner with alkaline phosphatase expression and biomineralization, including bone nodule formation and proliferation in preosteoblastic mouse calvaria cells.[15] Our results of positive effects of zinc on alkaline phosphatase activity and expression in bone forming cells also agree with their results. Also, it has been recently reported that zinc transporters, ZnT5 and ZnT7, are required for the activation of alkaline phosphatases and these two zinc transporters are responsible for loading zinc to alkaline phosphatases that are exposed to the extracellular site.[18] This study confirmed that zinc contributes to the whole formation of alkaline phosphatase.

The common histological assays to determine extracellular matrix (ECM) mineralization are such as von Kossa stain for phosphate group and Alizarin red S stain for calcium. Cell-matrix interactions are crucial for the regulation of cytoskeletal structure, growth and differentiation.[17] Cell survival depends on cellular interaction with extracellular matrix (ECM), with other cells and with soluble growth factors in the serum.[18] In the present study, we measured extracellular matrix bone nodule formation and Ca deposits using von Kossa staining. In the study, cellular calcium level was increased as medium zinc level increased in a proportional way. Ca deposit by von Kossa stains was also increased in zinc concentration-dependent manner. There was an increase in the deposited Ca as medium zinc level increased as assayed by von Kossa staining

In the present study, interestingly, zinc induced the MC3T3-E1 cells to express more bone-forming related genes, such as alkaline phosphatase and osteocalcin. Osteocalcin, also known as bone Gla protein, is the most abundant non-collagenous protein in the extracellular bone matrix.[21-24] Secreted by osteoblasts, osteocalcin is a vitamin K- and vitamin D-dependent protein that also signals terminal osteoblast differentiation.[25-28] Bone resorption marker gene, parathyroid hormone, was not affected by zinc levels. We cannot explain the lower expression of osteopontin which is the bone forming marker gene in Zn+ (15 μM $ZnCl_2$). It can be speculated that each different gene may be expressed at different bone forming stage and our experimental condition was about at the early stage of bone formation, which gave different gene expression patterns among the several bone forming genes.

In conclusion, we report that zinc positively affects osteoblast cell proliferation, alkaline phosphatase activity and morphological expression, and extracellular bone nodule formation. Zinc also increased the bone forming gene (alkaline phosphatase and osteocalcin) expressions in osteoblastic MC3T3-E1 cells. The current work supports the beneficial effect of zinc on bone mineralization and bone-related gene expression. The study results also promote further study as to the molecular mechanism of zinc deficiency for bone formation and thus help design preventive strategies for zinc-deficient bone diseases.

Literature Cited

1) Heaney RT. Calcium bone health and osteoporosis. In Bone and Mineral Research, vol. 4, pp.255-301, editor Peck WA, Elsevier. New York, 1986
2) Beattie J, Avenell A. Trace element nutrition and bone metabolism. *Nutr Res Rev* 5:167-188, 1992
3) Yamaguchi M, Fukagawa M. Role of zinc in regulation of protein tyrosine phosphatase activity in osteoblastic MC3T3-E1 cells: zinc modulation of insulin-like growth factor-I's effect. *Calcif Tissue Int* 76:32-38, 2005
4) Conlan D, Korula R, Tallentire D. Serum copper levels in elderly patients with femoral-neck fractures. *Age and Ageing* 19:212-214, 1990
5) Lowe MN, Fraser WD, Jackson MJ. Is there a potential therapeutic value of copper and zinc for osteoporosis?

Proceedings Nutr Soc 61:181-185, 2002
6) Eberle J, Schindmayer S, Erben RG, Stangassinger M, Roth HP. Skeletal effects of zinc deficiency in growing rats. *J Trace Elem Med Biol* 13:21-26, 1999
7) Yoshikawa M, Suzuki K, Kajio T, Koshikawa M, Imai T, Matsumoto A. Quantitative analysis of alkaline phosphatase activity and mineralization of a clonal osteoblast-like cell MC3T3-E1. *J Hard Tissue Biol* 8:37-42, 1999
8) Kwun IS, Beattie JH. Gene expression profile of zinc-deficient, homocysteine-treated endothelial cells. *J Food Sci Nutr* 8:390-394, 2003
9) Langmade SJ, Ravindra R, Daniels P, Andrews GK. The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene. *J Biol Chem* 275:34803-34809, 2000
10) Bessey OA, Lowry OH, Brock MJ. A method for the rapid determination of alkaline phosphatase with fibecubic millimeters of serum. *J Biol Chem* 164:321-329, 1946
11) Lowry OB, Rosenbrough MJ, Farr AL, Rebar RW. Protein measurement with folin phenol reagent. *J Biol Chem* 193:255-260, 1951
12) Yamaguchi M, Yamaguchi R. Action of zinc on bone metabolism in rats. Increases in alkaline phosphatase activity and DNA content. *Biochm Pharmacol* 35:773-777, 1986
13) Herzberg M, Foldes J, Steinberg R, Menczel J. Zinc excretion in osteoporotic women. *J Bone Miner Res* 5:251-257, 1990
14) Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S. *In vitro* differentiation an dcalcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J Cell Biol* 96:191-198, 1983.
15) Owen TA, Aronow M, Shallhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS. Progressive development of the rat osteoblast phenotype in-vitro: reciprocal relationships in the expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J Cell Physiol* 143:420-430, 1990
16) Beck Jr Gr, Sullivan EC, Moran E, Zerler B. Relationship between alkaline phosphatase levels, osteopontin expression, and mineralization in differentiating MC3T3-E1 osteoblasts. *J Cell Biochm* 68:269-280, 1998
17) Storrie H, Stupp SI. Cellular response to zinc-containing organoapatitie: an in vitro study of proliferation, alkaline phosphatase activity and biomineralization. *Biomaterials* 26: 5492-5499, 2005
18) Suzuki T, Ishihara K, Magaki H, Matsurra W, Kohda A, Okumura K, Nagao M, Yamaguchi-Iwai Y, Kambe T. Zinc transporters, Znt5 and ZnT7, are required for the activation of alkaline phosphatases, zinc-requiring enzymes that are glycosylphosphatidylinositol-anchored to the cytoplasmic membrane. *J Biol Chem* 280:637-643, 2005
19) Jones PL, Schmidhauser C, Bissel MJ. Regulation of gene expression and cell function by extracellular mtrix. *Crit Rev Eukaryot Gene Exp* 3(2):137-154, 1993
20) Meredith Jr JE, Fazeli B, Shwartz MA. The extracellular matrix as a cell survival factor. *Mol Biol Cell* 4(9)953-961, 1993
21) Hunter GK, Goldberg HA. Nucleation of hydroxyapatite by bone sialoprotein. *PNAS* 90:8562-8565, 1993
22) Hunter GK, Poitra MS, Underhill TM, Grynpas MD, Goldberg HA. Induction of collagen mineralization by a bone sialoprotein-decorin chimeric protein. *J Biomed Mater Res* 55:496-502, 2001
23) Yahai T, Katagiri T, Akiyama S, Imada M, Yamashita T, Chiba H, Takahashi N, Suda T. Expression of mouse osteocalcin transcripts, OG1 and OG2, is differently regulated in bone tissue and osteoblast cultures. *J Bone Miner Metab* 19:245-251, 2001
24) Tye CE, Rattray KR, Warner KJ, Gordon JA, Sodek J, Hunter GK, Goldberg HA. Delineation of the hydroxyapatite-nucleating domains of bone sialoprotein. *J Biol Chem* 278(10):7949-7955, 2003
25) Desbois C, Hogue DA, Karsenty G. The mouse osteocalcin gene cluster contains three genes with two separate spatial and temporal patterns of expression. *J Biol Chem* 269(2): 1183-1190, 1994
26) Harris NL, Rattray KR, Tye CE, Underhill TM, Somerman MJ, D'Errico JA, Chambers AF, Hunter GK, Goldberg HA. Functional analysis of bone sialprotein: identification of the hydroxyapatite-nucleating and cell-binding domains by recombinant peptide espression and site-directed mutagenesis. *Bone* 27(6):795-802, 2000
27) Lee AL, Hodges S, Eastell R. Measurement of osteocalcin. *Ann Clin Biochem* 37:432-446, 2000
28) Hauschka PV, Lian JB, Cole DE, Gundberg CM. Osteocalcin and matrix Gla protein: vitamin k-dependent proteins in bone. *Physiol Rev* 69(3):990-1035, 1989