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Role of zinc in cellular zinc trafficking and mineralization in a murine osteoblast-like cell line

Masashi Nagata[a,b,*,1], Bo Lönnerdal[b,1]

[a]Nutrition Research Department, Food Science Institute, Meiji Dairies Corporation, Odawara, Kanagawa 250-0862, Japan
[b]Department of Nutrition, University of California, Davis, CA 95616, USA

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Abstract

Zinc (Zn) supplementation stimulates bone growth in Zn-deficient humans and animals. A biphasic pattern of mineralization has been observed in cultured osteoblasts; an initiation phase and a progression phase. We used MC3T3-E1, a murine osteoblastic cell line, to elucidate the physiological role of Zn in osteoblast mineralization and cellular Zn trafficking during the mineralization event. Cells were cultured in media containing Chelex-treated fetal bovine serum and 1, 4, 10 and 20 μM Zn as $ZnSO_4$ for 14 days (early phase of mineralization) or 21 days (mid-to-late phase of mineralization). During the early phase of mineralization, Alizarin Red staining indicated that mineralization was increased by Zn in a dose-dependent manner. Although Zn exposure did not affect monolayer Zn concentration, metallothionein (MT) mRNA expression increased dose-dependently as assessed by real-time PCR. During the late phase of mineralization, mineralization was maximal at 1 μM Zn and monolayer Zn concentration reflected Zn exposure. The increase in MT mRNA expression during the late phase was similar to that during the early phase, but the difference in expression between culture Zn concentrations tended to be smaller. ZnT-2 mRNA expression decreased significantly with increasing zinc concentrations in the culture medium during the early phase, but increased significantly during the late phase. Osteocalcin mRNA levels were positively correlated to Zn exposure at both time points. Taken together, we propose that Zn may play an important role in osteoblast mineralization through Zn trafficking involving Zn storage proteins and Zn transporters.
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Keywords: Bone; Zinc; Osteoblasts; Mineralization; Zinc transporters; Metallothionein

1. Introduction

Bone is synthesized by precise cell-mediated mechanisms, starting within matrix vesicles secreted from osteoblasts and chondrocytes. Two patterns of mineral deposition on an extracellular matrix have been described [1]: (i) matrix vesicle-mediated mineral initiation and (ii) heterogeneous nucleation of mineral crystals on collagen and other extracellular matrix proteins. Osteocalcin, otherwise known as bone gla-protein, is a bone-specific non-collagenous extracellular matrix protein, which is post-translationally modified by the action of vitamin K-dependent γ-carboxylases. Production of dicarboxylic glutamyl residues enhances calcium binding [2]. Osteocalcin has an inhibitory function of mineral deposition and osteocalcin-deficient mice have increased bone mineral density compared with normal mice [3]. In cultured osteoblasts, osteocalcin is regarded as a mineralization marker.

Zinc (Zn) is an important regulator of biological functions and is a constituent of various enzymes and proteins. Zn plays important roles in bone metabolism. Animal studies have shown that Zn deficiency causes low bone mass [4]. In humans, low serum Zn and low bone turnover were reported in small-for-gestational age preterm infants [5] and low Zn intake has been reported to be associated with low bone mass in adult women [6]. Inadequate dietary intake of Zn causes a decrease in number of osteoblasts [7], and in vitro studies show that Zn augments DNA synthesis in murine osteoblast-like cells in a dose-dependent manner [8]. Zn is also involved in the stimulation of collagen production in rat calvaria [9]. Studies have shown that Zn has an inhibitory effect on bone resorption [10] as well as a stimulatory effect on bone formation and mineralization in osteoblast cell cultures.

Cellular trafficking of Zn is controlled by Zn storage proteins and Zn transporters (ZnTs). Metallothioneins (MTs) are cellular metal storage proteins induced by essential trace elements such as Zn [11,12] and Cu [13] and toxic metal ions [14] such as Cd and Hg. MT levels may vary with age and type of tissue and depend upon nutritional and physiological factors. MT synthesis occurs primarily in organs involved in absorption and excretion. At the cellular level, MT is mainly distributed to the cytoplasm and to a lesser extent in the nuclei and lysosomes [15].

* Corresponding author. Nutrition Research Department, Food Science Institute, Meiji Dairies Corporation, Odawara, Kanagawa 250-0862, Japan. Tel.: +81 465 37 3661; fax: +81 465 37 3624.
E-mail address: masashi_nagata@meiji-milk.com (M. Nagata).
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A number of mammalian proteins that participate in Zn trafficking across membranes have been described [16]; these are divided into two distinct families. The ZnT (solute-linked carrier 30 (SLC30A)) proteins decrease intracellular Zn by mediating Zn efflux from cells or influx into intracellular vesicles. The Zip [Zrt- and Irt-like proteins (SLC39A)] proteins promote Zn transport from the extracellular fluid or from intracellular vesicles into the cytoplasm. Association of zinc transporters on bone biology has been suggested. In our study, five zinc transporters, ZnT-1, ZnT-2, ZnT-4, ZnT-5 and Zip3, were chosen to evaluate the role of cellular zinc trafficking in osteoblast mineralization. Kelleher et al. [17] demonstrated that the association between Zn content in milk and specific changes in gene expression and protein levels of ZnT-1, ZnT-2, ZnT-4 and Zip3 using rat mammary gland and human mammary cell line. Osteoblasts have a characteristic secretory function with regard to matrix vesicle secretion as well as mammary cells with milk-secretion function. An essential role for ZnT-5 [18] in bone mineralization or differentiation of osteoblasts has been reported.

Although there are some reports on the relationship between Zn transporters, MT and bone status, it remains to be elucidated the mechanistic role of Zn transporters and metallothionein in osteoblastic mineralization. In the present study, to clarify the physiological role of Zn in osteoblastic mineralization and in cellular Zn trafficking during the mineralization event, we applied in vitro experiments using MC3T3-E1, a murine osteoblastic cell line.

2. Materials and methods

2.1. Cell culture

MC3T3-E1 (Riken Cell Bank, Tsukuba, Japan), mouse pre-osteoblastic cells, were sub-cultured every three days in α-Modified Minimum Essential Medium (α-MEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS). Passages 10–16 of subculture were used for all experiments.

2.2. Chelex-treatment of culture media

To remove Zn from the culture medium, FBS was treated with Chelex 100-resin (Chelex; BioRad, Hercules, CA, USA), according to the product instruction, then mixed with 50 μg/ml of ascorbic acid and 10 mM of β-glycerophosphate-containing α-MEM. Other trace minerals, such as copper and iron, are thought to potentially affect bone formation [19,20], and concentrations of these two minerals in FBS were reduced by Chelex treatment. Therefore, the Chelex-treated FBS containing media were supplemented with copper and iron to a concentration of 0.4 μM Cu (as $CuSO_4$) and 6 μM Fe (as $FeSO_4$), respectively, which are the same concentrations as those of non-treated FBS-containing medium. Four different concentrations of Zn were used in the experimental media: 1, 4, 10 and 20 μM (added as $ZnSO_4$).

2.3. Mineralized nodule formation analysis

MC3T3-E1 cells were seeded in 10% FBS-containing α-MEM at 1×10^5 cells per well in 12-well culture plates. After reaching 70–80% confluency, the medium was changed to 10% Chelex-treated FBS-containing medium with various concentrations of Zn. On days 14 and 21 post-seeding, representative time points for early and late stage of mineralization in osteoblast culture [21], cells were stained by Alizarin Red S and quantified bound calcium by the method of Stanford et al. [22] with slight modifications. Briefly, the medium was aspirated from the wells, and cells were rinsed twice with phosphate-buffered saline (PBS). The cells were fixed with ice-cold 70% (v/v) ethanol for 1 h. Ethanol was then removed, and cells were rinsed twice with deionized water. Cells were stained with 40 mM Alizarin Red S in deionized water (pH 4.2) for 10 min at room temperature. The Alizarin Red S solution was removed by aspiration, then cells were rinsed with deionized water and washed in PBS while rocking for 15 min at room temperature. PBS was removed, followed by rinsing with PBS, and destaining for 15 min with 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0). The extracted stain was transferred to a 96-well plate, and the concentration of Alizarin Red S in each sample was quantified by comparing the absorbance with those from Alizarin Red S standards. The absorbance at 540 nm was measured by Multiskan Ascent (Labsystems, Helsinki, Finland). In the method by Stanford et al. [22], absorbance was measured at 562 nm, but our reader used a wavelength of 540 nm which yields results very similar to those at 562 nm. Mineralization values were normalized to the protein concentration of the live cells corresponded to each samples by BCA Protein Assay kit (Pierce, Rockfield, IL, USA).

2.4. Total RNA extraction and real-time reverse transcriptase-polymerase chain reaction analysis

On days 14 and 21 post-seeding, total RNA from cultured cells was extracted with TRIzol (Invitrogen) according to the manufacturer's protocol. One microgram of total RNA from each sample was subjected to reverse transcription with oligo (dT) primers (Applied Biosystems, Foster City, CA, USA). Real-time polymerase chain reaction (PCR) reactions were then carried out in a 25 μl of reaction mixture (1.5 μl of cDNA, 12.5 μl of SYBR Green PCR Master Mix (Applied Biosystems), 5.0 μl of 10 μM specific gene primer pair and 6 μl of H_2O) in ABI Prism 7900 Sequence Detection System (Applied Biosystems). Sequences of the primers used in the real-time PCR reaction were as follows: osteocalcin: forward: 5′-ACGGTATCACTATTTAGGACCTGTG-3′, reverse: 5′-ACTTTATTTTGGAGCTGCTGTGAC-3′; MT-I: forward: 5′-CCGGACCAACTCAGAGTCTTG-3′, reverse: 5′-TTCACATGCTCGGTAGAAAACG-3′; Zn transporter 1 (ZnT-1): forward: 5′-GAAGAAGATAGGGCTGGACAACTT-3′, reverse: 5′-CCCAAGGCATCTCCAAGGA-3′; Zn transporter 2 (ZnT-2): forward: 5′-CGTCCGAGCTGCCTTCAT-3′, reverse: 5′-GCCACTAGGACACCCATGCT-3′; Zn transporter 4 (ZnT-4): forward: 5′-TTTCGCGCTTTAATAAACTTCGA-3′, reverse: 5′-GGTGCGCCCCGTTCA-3′; ZnT 5 (ZnT-5): forward: 5′-GCTCTGCTCTTTGGAAACTTCTG-3′, reverse: 5′-CCTGGTGTGCTGCTCTGTTC-3′; Zip3: forward: 5′-AACAGCATGTCAGCTTCTCCTATG-3′, reverse: 5′-GGATCCCGCCTGCACTAA-3′; glyceraldehyde phosphate dehydrogenase (GAPDH): forward: 5′-TGCCAAGTATGATGACATCAAGAAG-3′, reverse: 5′-AGCCCAGGATGCCCTTTAGT-3′. The PCR program was initiated for 2 min at 50°C and 10 min at 95°C before 40 thermal cycles, each of 15 s at 95°C and 1 min at 60°C, and then 15 s at 95°C, 15 s at 60°C and 15 s at 95°C. Data were analyzed according to the comparative cycle threshold (Ct) method [23] and were normalized by GAPDH expression in each sample.

2.5. Protein extraction and Western Blotting for MT

At days 14- and 21-post-seeding, cellular protein was extracted with cell lysis buffer (pH 8.0) containing 100 mM Tris(hydroxymethyl)aminomethane,100 mM NaCl, 0.5% Triton X-100 and protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Equal amounts of osteoblast protein (100 μg) were diluted to 1:1 in Laemmli sample buffer with 1% β-mercaptoethanol and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%). Proteins in the gel were transferred to nitrocellulose membrane for 90 min at 350 mA. Blots were blocked overnight at 4°C with 5% nonfat milk in PBS with 0.1% Tween-20 (PBST) and washed 3 times in PBST. Blots were incubated with a mouse monoclonal antibody to MT (specific against MT-I and II, 1:2000 in PBST, Dako, Carpinteria, CA, USA) for 45 min and washed three times in PBST. Blots were incubated with sheep-anti-mouse IgG conjugated to horseradish peroxidase (Dako). Blots were visualized with chemiluminescence (SuperSignal Femto, Pierce) and quantified using the Chemi-doc Gel Quantification System (BioRad). Blots were stripped and re-probed for β-actin as a loading control.

2.6. Analysis of monolayer Zn

On Days 14 and 21 post-seeding, Zn concentrations were measured in cultured monolayers. After one wash of PBS, the monolayer was scraped off by a cell scraper, followed by digestion in 200 μl of ultra-pure 16 N nitric acid, dilution to 1 ml with deionized H_2O and digestion overnight. Zn concentration was determined by flame atomic absorption spectroscopy (Solaar Model M6, Thermo Jarrell Ash, Franklin, MA, USA). The value was normalized to the protein concentration of the monolayer cells as analyzed by the BCA Protein Assay.

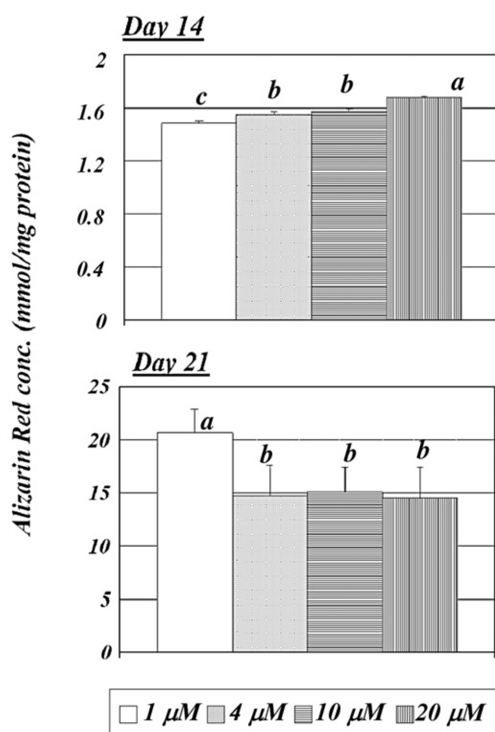
2.7. Statistics

All experiments were carried out at least twice with $n=6$ per group. Data are presented as means±S.D.. Statistical analysis was performed using StatView (Cary, NC, USA). For mineralization assays and monolayer Zn measurements, two-way analysis of variance (ANOVA) was performed with variables for Zn dose and mineralization period as time, and one-way ANOVA was performed with variables for Zn dose regarding expression of mRNA and protein. Sheffe's method, a post hoc test, was performed at each time point, and $P<.05$ was regarded as significantly different.

3. Results

3.1. Mineralization of MC3T3-E1 osteoblast cells

The results from quantitative analysis of osteoblast mineralization are shown in Fig. 1A. Mineralization increased in a time-dependent manner (two-way ANOVA; time as variable, $P<.0001$). The effect of Zn exposure on mineralization was quite different between the two time points (two-way ANOVA; dose as variable,



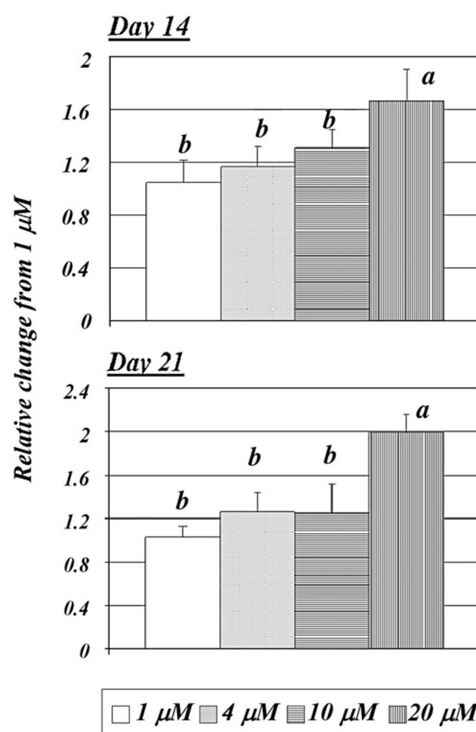


Fig. 1. (A) Changes in mineralization of MC3T3-E1 cells with zinc exposure. Cells cultured in varying Zn concentrations (1, 4, 10, 20 μM) were stained by Alizarin Red S on days 14 and 21. Mineralization values were normalized to the protein concentration of the live cells. Data are presented as means±S.D. Means with different letters are significantly different as analyzed by two-way ANOVA followed by Sheffe's method as a post hoc test at each time point ($P<.05$). (B) Relative changes in osteocalcin mRNA expression with zinc exposure in cultured MC3T3-E1 cells. Osteocalcin mRNA was measured using real-time PCR and normalized by mRNA expression of GAPDH. Data are presented as means±S.D. of osteocalcin transcripts in d14 and d21 samples in response to varying Zn concentrations (1, 4, 10, 20 μM). Means with different letters are significantly different as analyzed by one-way ANOVA followed by Sheffe's method as a post hoc test at each time point ($P<.05$).

$P<.0001$, interaction between dose and time, $P<.0001$). In the early phase of culture (Day 14), mineralization was promoted by increasing concentrations of Zn in the culture medium. In contrast, during late phase culture (Day 21), maximal mineralization was found in the group treated with the lowest concentration of Zn (1 μM), and similar mineralization was observed for the other three treatment groups, ranging from 4 μM Zn (the Zn concentration of FBS-added culture medium) to 20 μM Zn, the physiological concentration of Zn in murine serum [24].

3.2. Osteocalcin mRNA expression in MC3T3-E1 cells

Gene expression of osteocalcin (Fig. 1B) increased significantly with increased Zn exposure ($P=.0010$) and expression was the highest in the 20 μM Zn group. These tendencies were similar, and not significantly different between the two culture times.

3.3. Zn concentration of monolayer culture of MC3T3-E1 cells

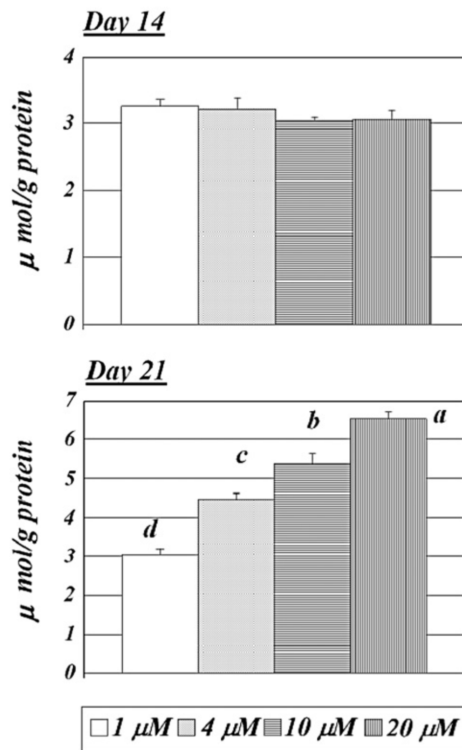
Zn contents of the culture wells are shown in Fig. 2A. Values are expressed as Zn content per protein content, and include the intra- and extracellular Zn contents within the culture well. In the early phase, no significant change was found between the various concentrations of Zn in the culture medium. However, in the late phase, Zn content increased notably, in a dose- and time- dependent manner (two-way ANOVA; dose and time as variables, $P<.0001$ and $P<.0001$, respectively, interaction between dose and time, $P<.0001$). The 20-μM Zn group had more than twice the Zn content of the control group.

3.4. Messenger RNA and protein expression of MT in MC3T3-E1 cells

Gene expression of MT-I (Fig. 2B) increased dose-dependently with increasing Zn exposure ($P<.0001$). During the early phase, MT-I expression in the 10 and 20 μM Zn groups was higher than in the lower Zn concentration groups. During the late phase, while the higher expression in the 20 μM Zn group remained, expression in the 10 μM Zn group was comparable to those in the lower Zn concentration groups. Protein expression of MT-I was not significantly changed by varying Zn concentration (data not shown).

3.5. Zn transporter mRNA expression in MC3T3-E1 cells

We observed different responses in relative gene expression to Zn exposure among the five Zn transporters we analyzed. There was no significant difference in Zip3 expression (Fig. 3A) between different levels of Zn exposure. ZnT-1 expression (Fig. 3B) changed significantly with different Zn concentrations in the culture medium during the early phase, but there was no overall significant change between Zn treatments by two-way ANOVA. ZnT-2 expression (Fig. 3C) showed significant dose-dependent changes ($P=.0396$). ZnT-2 expression decreased significantly with increased Zn concentration in the culture medium during the early phase, but increased significantly during the late phase. ZnT-4 expression (Fig. 3D) tended to be influenced by Zn exposure. During early culture, ZnT-4 expression was higher in the 20 μM Zn group than that in the control group, but there was no change in ZnT-4 expression between all the groups during late culture. ZnT-5 expression was



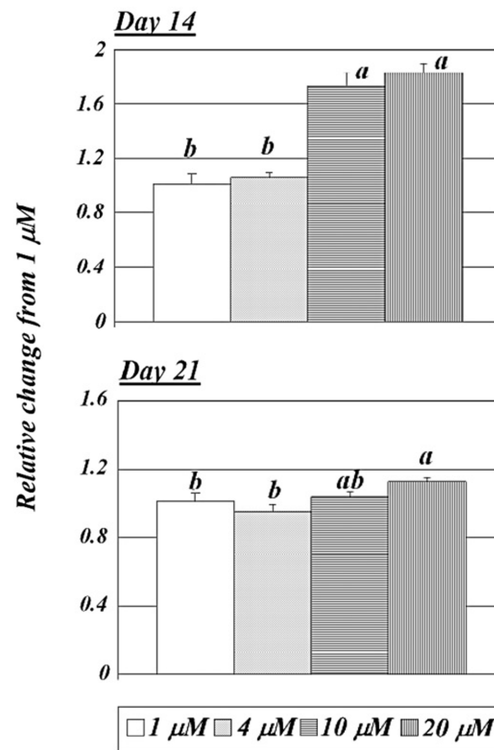


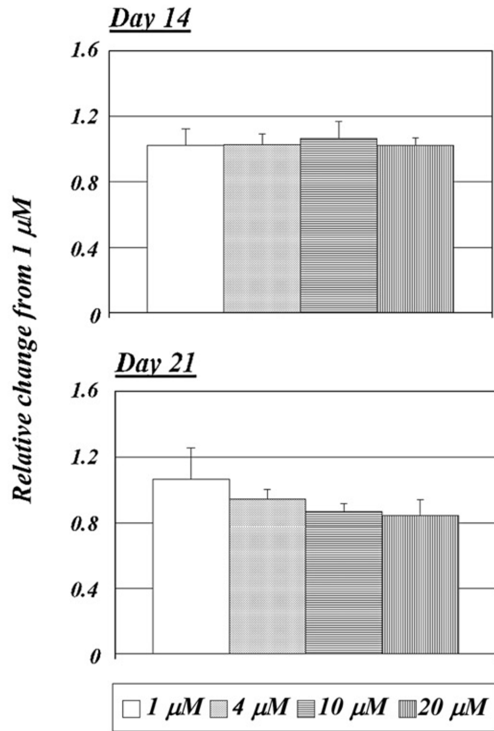
Fig. 2. (A) Changes in zinc concentration of monolayer cultured MC3T3-E1 cells with zinc exposure. Cells cultured in varying Zn concentrations (1, 4, 10, 20 μM) were scraped on Days 14 and 21, followed by digestion. Zinc concentrations of the digested samples were determined by flame atomic absorption. Values were normalized to the protein concentration. Data are presented as means±S.D. Means with different letters are significantly different as analyzed by two-way ANOVA followed by Sheffe's method as a post hoc test at each time point ($P<.05$). (B) Relative changes in metallothionein mRNA expression in cultured MC3T3-E1 cells with zinc exposure. Metallothionein mRNA was measured using real-time PCR and normalized by mRNA expression of GAPDH. Data are presented as means±S.D. of metallothionein transcripts in d14 and d21 samples in response to varying zinc exposure (1, 4, 10, 20 μM). Means with different letters are significantly different as analyzed by one-way ANOVA followed by Sheffe's method as a post hoc test at each time point ($P<.05$).

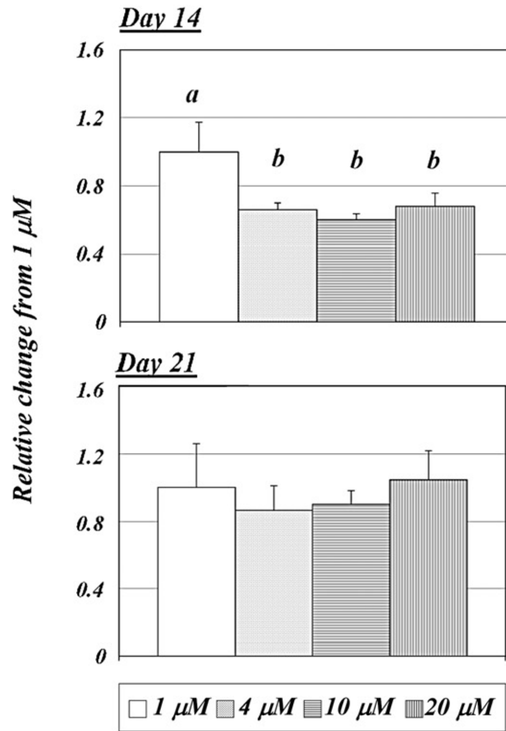
not changed significantly with Zn exposure or with culture time (data not shown).

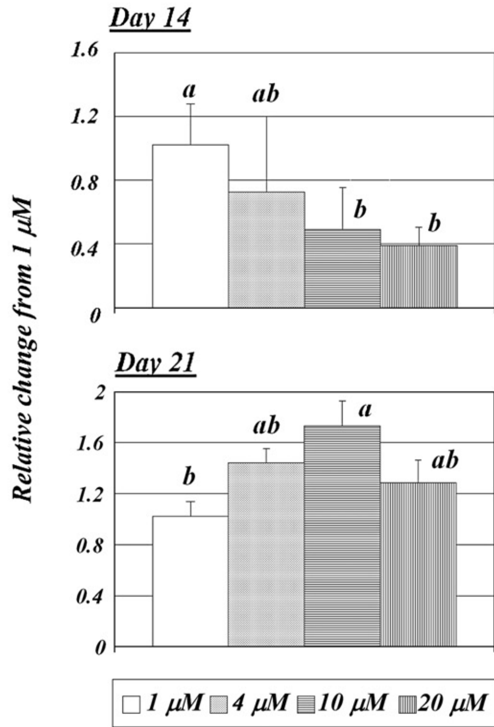
4. Discussion

The present study was conducted to determine effects of Zn exposure on osteoblast mineralization in early- and late-mineralized MC3T3-E1 osteoblast-like cells. We also evaluated the cellular accumulation of Zn and expression of Zn transporters and MT during the mineralization process. In our study, osteoblast mineralization was promoted dose-dependently by Zn exposure during the early phase. With regard to physiological significance, osteoblast mineralization during the early phase was dose-dependently increased, especially from the 4 to the 10-μM Zn groups, corresponding to serum Zn concentrations of marginally Zn-deficient animals [25]. In the late phase, maximal mineralization was observed in the 1 μM Zn group. Since 1 μM is a very low Zn concentration, cellular mineralization observed during the late phase culture appears to be limited in our in vitro system. During the late phase, mineralization per protein remained lower from 4 to 20 μM Zn compared to that of 1 μM Zn. However, because the protein concentration of the cultured cells increased significantly time- and dose-dependently in our study (data not shown), the actual amount of mineralization per whole culture, which is a representation of bone tissue mineralization in our model, was increased in the high Zn concentration group during the late phase. This suggests that mineralization of individual cells seemed to be terminated properly but mineralization of the whole cell culture appears to be increased in response to higher Zn concentrations. Therefore, both in early and late phases, this suggests that Zn exposure of osteoblasts in culture is critical for mineralization.

The bone mineralization process has been clarified by in vivo and in vitro studies on matrix vesicles from cartilage and bone, and mainly consists of two phases [1]. The maximal concentration of Zn used in this study is similar to the physiological serum Zn level in mice. Extracellular matrix production and extracellular environment in osteoblast have been reported to be involved in the mineralization of osteoblasts [26]. Therefore, it is reasonable to consider two factors: extracellular matrix production and Zn concentration. During the early phase of osteoblast differentiation, alkaline phosphatase is believed to be highly expressed. Anderson et al. [27] concluded that the early phase of mineralization was closely associated with tissue non-specific alkaline phosphatase. According to a previous report [28], addition of Zn to bone culture resulted in a concentration-dependent increase in alkaline phosphatase activity. Although we did not evaluate the alkaline phosphatase activity in our study, we hypothesize that the differences in mineralization of osteoblasts with Zn exposure during the early phase may partly be due to cellular alkaline phosphatase activity. We found that osteocalcin gene expression increased with increasing Zn concentration of the medium. Osteocalcin has been regarded as a mineralization marker in osteoblast culture [26]. Expression of osteocalcin begins during the early phase of mineralization and is promoted during the mineralization process. Osteocalcin has also been considered as a termination factor of mineralization, which is supported by the observation that







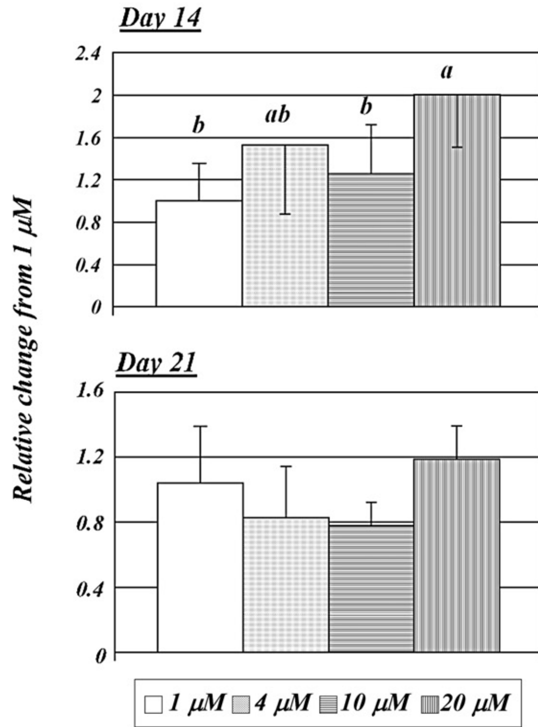


Fig. 3. Relative changes in mRNA expression of (A) Zip3, (B) ZnT-1, (C) ZnT-2 and (D) ZnT-4 in MC3T3-E1 cell culture with zinc exposure. Zip3, ZnT-1, -2 and -4 mRNA were measured using real-time PCR and normalized by the mRNA of GAPDH. Data are presented as means±SD of Zip3, ZnT-1, -2 and -4 transcripts in the 14d and 21d samples at varying zinc concentration exposure (1, 4, 10, 20 μM). Means with different letters are significantly different as analyzed by one-way ANOVA followed by Sheffe's method as a post-hoc test at each time point ($P<.05$).

osteocalcin knockout mice develop higher bone mass [3,29]. According to a recent report on osteosclerosis, a disease of excessive bone mineralization, osteocalcin expression was reduced due to immature differentiation of osteoblasts [30]. In our study, the lower osteocalcin expression and the higher mineralization found during the late phase culture in the 1 μM Zn group seems to have symptomatic similarity to osteosclerosis, which might suggest the existence of immature osteoblasts in 1 μM Zn culture. Judging from our present findings, osteocalcin may promote appropriate differentiation and proper termination of the mineralization stage. Taken together the past and present findings regarding extracellular matrix production by osteoblasts, physiological levels of Zn may induce appropriate differentiation of osteoblasts during the mineralization stage. A direct inhibitory effect of Zn on the hydroxyapatite formation might be possible, because addition of Zn has been shown to inhibit the deposition of amorphous calcium phosphate at concentrations less than 100 μM [31,32]. However, the different manners of mineralization found between the two time points in our study could not be ascribed only to Zn concentration.

In the present study, we evaluated Zn content of the cultured cells to elucidate the role of Zn accumulation in the development of osteoblast mineralization. Although Zn accumulation seems to be unaffected by Zn exposure in the early phase of culture, Zn content per well was clearly increased with Zn exposure during the late phase. Zn content of the culture well reflects intra- and extracellular Zn contents; thus, it is technically difficult to distinguish between intra- and extracellular Zn. From our present results on protein concentration, extracellular matrix production appears to be promoted during late phase culture. Therefore, the dose-dependent increase in culture Zn content in the late phase suggests binding of Zn to the extracellular bone matrix which is secreted during differentiation in osteoblast cultures. Despite the increased mineralization, Zn accumulation per well in the early phase remained similar for varying concentrations of Zn, which may mean that the osteoblast mineralization process is regulated by another mechanism, particularly with regard to cellular Zn movements.

Expression of MT-I mRNA increased with increasing Zn concentrations at both time points. Zn exposure has been shown to augment MT-I expression in a dose-dependent fashion in various mammalian cells [33,34]. In our study, the tendency for change in MT-I mRNA expression was similar at the two time points but appeared to change in response to mineralization stage, as intracellular Zn storage was more pronounced at higher Zn concentrations in the early phase, whereas the difference in expression in response to Zn exposure was smaller in the late phase. These results suggest that intracellular Zn storage is associated with the expression of MT-I as mineralization proceeds. In our study, we found no change in MT-I protein expression with varying zinc concentration. The monoclonal antibody used in our study was specific against both MT-I and MT-II. Recently, Fong et al. [35] demonstrated that mRNA expression of MTs-I and -II was induced in the growth plate of wild-type mice fed a Zn-limited diet. The difference in our results between the expression of protein and mRNA might be partly ascribed to MT-II expression.

We evaluated mRNA expression of Zn transporters. Except for ZnT-5 and Zip3, expression of the other Zn transporters, ZnT-1, ZnT-2, and ZnT-4, changed with varying concentrations of Zn in osteoblast culture. Further, the expression patterns for these transporters differed between the two time points. In particular, ZnT-2 mRNA expression may be mineralization stage- and Zn concentration-dependent. ZnT-2 has been reported to be localized to the endosome in mammary epithelial cell [36]. Chowanadisai et al. [37] reported a role for ZnT-2 in Zn secretion in mammary epithelial cells as studied by gene knockdown of ZnT-2. ZnT-2 has also been shown to have a vesicular localization in pancreatic acinar cells [38]. The osteoblast has a characteristic secretory function, as well as mammary cells and pancreatic acinar cells, with regard to matrix vesicle secretion during the mineralization process. A role for ZnT-2 in bone cells has not been reported previously. If ZnT-2 in osteoblasts also plays a role as Zn supplier from the intra- to the extracellular environment similar to that of mammary epithelial cells, the time- and dose-dependent manner of mineralization with Zn exposure might be partly associated with ZnT-2 expression via the secretion of Zn. Knowledge of the localization of ZnT-2 in matrix vesicles during the mineralization process would help to elucidate the role of ZnT-2 in the promotion of osteoblast mineralization. In our study, ZnT-1 and ZnT-4 expression also changed during the mineralization process, but did not seem to directly respond to Zn concentration in the medium. Those proteins might be involved, but indirectly, in osteoblast mineralization. Although a role for ZnT-5 in bone mineralization or differentiation of osteoblasts has been reported [18], we observed no change in mRNA expression of ZnT-5 under various concentrations of zinc exposure. The present study did not elucidate the role of Zn transporters like Zips in Zn import from the extracellular environment. Since several Zn transporters have been identified in mammalian cells [16,39], it seems likely that other Zn transporters exist and act redundantly in osteoblast mineralization. Taken together with our present findings about MT and Zn transporters, we speculate that Zn is secreted from osteoblasts as a progression of the mineralization stage. Recently, Bobilya et al. [40] reported that ZnT-2 and MT are presumably performing a similar function in cells of the blood-brain barrier, and sequestering Zn that has been withdrawn from the brain. Further studies on the relationship between cellular Zn movement and mineralization need to be conducted using other techniques such as molecular imaging in order to investigate actual cellular Zn trafficking during different stages of mineralization. In our study, gene expression of proteins critical for bone mineralization was investigated. However, according to a report regarding Zn transporters in mammary cells [17], post-transcriptional or post-translational regulation of zinc transporters may play a role in regulating milk Zn concentration. Therefore, not only protein expression but also subcellular localization of Zn transporters needs to be evaluated by another experiment to elucidate actual role of Zn transporters in osteoblast mineralization. We found significant increases in osteocalcin and MT mRNAs although Zn content in the early monolayer culture remained similar with varying Zn concentrations. We speculate that intracellular Zn movement mediated by Zn transporters, especially translocation of Zn into the nucleus for modifying transcription may explain the modification in transcription of these genes. Even though indistinguishable between intra- and extracellular zinc content on Day 21, intracellular mechanisms (i.e., intracellular Zn movement) and/or some transcriptional modification of extracellular matrices are suggested to play some role in MT expression. A recent study suggests a role for Zip13 in cell signaling and connective tissue development, including maturation of osteoblasts [41]. Thus, it is possible that other Zn transporters may be involved in bone mineralization.

In conclusion, we have shown that Zn may play an important role in osteoblast mineralization through intra-and extracellular Zn movements involving a Zn storage protein (MT) and that Zn transporters might mediate the effects of Zn on osteoblast mineralization. These findings provide mechanistic insights into the nutritional role of Zn in bone formation and bone mineralization.

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