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# Effect of β-alanyl-L-histidinato zinc on differentiation of osteoblastic MC3T3-El cells: Increases in alkaline phosphatase activity and protein concentration

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## Abstract

The effect of β-alanyl-L-histidinato zinc (AHZ) on bone cell function was investigated in osteoblastic MC3T3-E1 cells. Cells were cultured for 3 days at 37° C in a $CO_2$ incubator in plastic dishes containing α-modified minimum essential medium supplemented with 10% fetal bovine serum. After the cultures, the medium was exchanged for that containing 0.1% bovine serum albumin plus AHZ ($10^{-7}$–$10^{-5}$ M) or other reagents, and the cells were cultured further for appropriate periods of time. The presence of AHZ ($10^{-7}$–$10^{-5}$ M) produced a remarkable increase of alkaline phosphatase activity and protein concentration in osteoblastic cells. Thus increases were seen with the prolonged cultivation (12–21 days). With the culture of 1, 3 and 12 days, the effect of AHZ ($10^{-6}$ M) to increase alkaline phosphatase activity and protein concentration was more intensive than the effect of zinc sulfate ($10^{-6}$ M). The AHZ effects were completely abolished by the presence of cycloheximide ($10^{-6}$ M), indicating that AHZ stimulates protein synthesis in the cells. The present study suggests that AHZ has a stimulatory effect on cell differentiation, and that this effect is partly involved on protein synthesis in osteoblastic cells. (Mol Cell Biochem **131:** 19–24, 1994)

*Key words:* β-alanyl-L-histidinato zinc, bone metabolism, cell differential effect, protein synthesis, osteoblastic cells

## Introduction

Zinc has been demonstrated to serve a wide variety of functions in the mammalian system, and is essential for growth in humans and many animals [1]. Bone growth retardation is a common finding in various conditions associated with zinc deficiency [2, 3]. Recently, it has been demonstrated that zinc has a stimulatory effect on bone formation and mineralization *in vivo* [4] and *in vitro* [5]; the metal stimulates bone protein synthesis, which is a cellular mechanism [6]. Thus, zinc plays a physiologic role as an activator in the regulation of bone formation.

On the other hand, β-alanyl-L-histidinato zinc (II), in which zinc is chelated to β-alanyl-L-histidine, is a new compound with a molecular weight of 289.61. More re-

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cently, it has been reported that β-alanyl-L-histidinato zinc (AHZ) can stimulate bone formation and calcification *in vivo* [7] and *in vitro* [8]. At the same dosage as zinc, AHZ has a more intensive effect on bone metabolism than zinc sulfate [7, 8], suggesting that the mode of AHZ action differs from that of zinc sulfate.

As a possible cellular mechanism, it has been found that AHZ can increase proliferation and DNA content in osteoblastic cells and that this effect may be dependent on protein synthesis [9]. Those effects were observed with the cultivation of a short term. Furthermore, the present investigation was undertaken to clarify whether AHZ can increase alkaline phosphatase activity and protein concentration, as a marker of cell differentiation, in osteoblastic cells with the prolonged cultivation. It was found that AHZ may have a stimulatory effect on the differentiation of osteoblastic cells.

## Materials and methods

*Chemicals*

AHZ (Zeria Pharmaceutical Co., Tokyo, Japan) was dissolved in 1 N HCl solution and adjusted to pH 7.0 with 1 N NaOH solution to concentrations ranging from $10^{-5}$–$10^{-3}$ M. α-Modification of Eagle's minimum essential medium (α-MEM) was obtained from Flow Laboratories, Inc. (McLean, Va, USA). Fetal bovine serum was obtained from Bioproducts Inc. (Walkersvile, Md., USA). Bovine serum albumin (fraction V) and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Tissue culture plastic dishes were purchased from Falcon Plastics (Los Angeles, Calif, USA). Other materials used were commercial products of the highest grade available.

*Cell culture*

MC3T3-E1 cells [10] were generously provided by Drs. Y. Amagai and S. Kasai (Koriyama, Japan). The cells were cultured at 37° C in a $CO_2$ incubator in plastic dishes containing α-MEM supplemented with 10% fetal bovine serum (FBS). They were subcultured every 3 days using 0.2% trypsin plus 0.02% etylenediaminetetraacetic acid (EDTA) in $Ca^{2+}$-, $Mg^{2+}$-free phosphate-buffered saline (PBS). For experiments, about $2.5 \times 10^4$ cells per dish were cultured for 3 days to obtain confluent monolayers in 35 mm plastic dishes containing 2 ml α-MEM with 10% FBS. After the cultures, the cells were rinsed with PBS, the medium was exchanged for that containing 0.1% bovine serum albumin (BSA) plus various concentrations of AHZ, zinc sulfate or other chemicals and the cells were cultured further for appropriate periods of time.

*Determination of cell numbers*

After trypsinization using 0.2% trypsine plus 0.02% EDTA in $Ca^{2+}$-, $Mg^{2+}$-free PBS, cell numbers were determined by the use of an electronic particle counter.

*Analytical procedures*

To assay alkaline phosphatase activity in cells after appropriate treatment periods, cells were washed 3 times with PBS, scraped into 0.5 ml of ice-cold 6.5 mM barbital buffer (pH 7.4) and disrupted for 60 s with an ultrasonic device. The supernatant centrifuged at 600 g for 5 min was used for the measurement of enzyme activity. The enzyme assay described below was carried out under optimal conditions. Alkaline phosphatase activity was determined by the method of Walter and Schutt [11]. The enzyme activity was expressed as nanomoles of p-nitrophenol liberated per minute per milligram protein.

To determine protein concentration in cells, osteoblastic cells were washed 3 times with PBS, scraped into 0.5 ml of ice-cold 6.5 mM barbital buffer (pH 7.4) containing 0.2% polyoxyethylene(10) octylphenyl ether (Triton X-100) solution and disrupted for 60 s with an ultrasonic device. Protein concentration in the homogenate of cells was determined by the method of Lowry *et al.* [12] and expressed as the amount of protein (micrograms) per dish.

To measure DNA content in the cells, the cells were detached by using 0.2% trypsin plus 0.02% EDTA in $Ca^{2+}$-, $Mg^{2+}$-free PBS and washed with PBS. The cells were shaken with 2.0 ml of ice-cold 0.1 N NaOH solution for 24 h after disruption [13]. After alkali extraction, the samples were centrifuged at 10,000 g for 5 min, and the supernatant was collected. DNA content in the supernatant was determined by the method of Ceriotti [14] and expressed as the amount of DNA (micrograms) per dish.

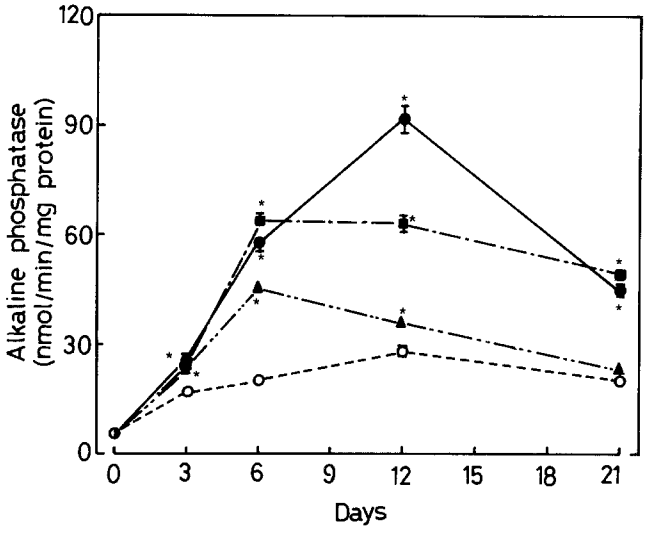


*Fig. 1.* Time-dependent effect of β-alanyl-L-histidinato zinc (AHZ) on alkaline phosphatase activity in osteoblastic MC3T3-E1 cells. Cells were cultured for 3, 6, 12 and 21 days in medium containing $10^{-7}$ to $10^{-5}$ M of AHZ. Each value is the mean ± SEM of 6 dishes. * $P < 0.01$, as compared with the control values. ○; Control, ▲; $10^{-7}$ M AHZ, ■; $10^{-6}$ M AHZ, ●; $10^{-5}$ M AHZ.

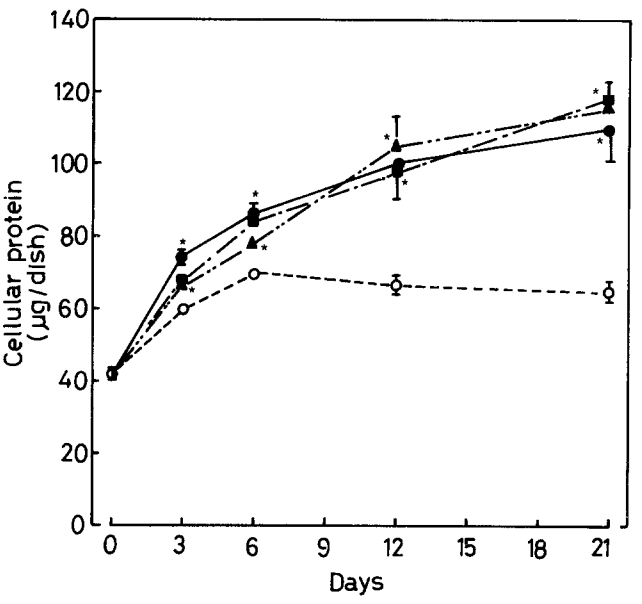


*Fig. 2.* Time-dependent effect of β-alanyl-L-histidinato zinc (AHZ) on protein concentration in osteoblastic MC3T3-E1 cells. Cells were cultured for 3, 6, 12 and 21 days in medium containing $10^{-7}$ to $10^{-5}$ M of AHZ. Each value is the mean ± SEM of 6 dishes. * $P < 0.01$, as compared with the control values. ○; Control, ▲; $10^{-7}$ M AHZ, ■; $10^{-6}$ M AHZ, ●; $10^{-5}$ M AHZ.

*Statistical methods*

Data are expressed as means ± SEM. Statistical differences were analyzed using Student's t-test; p values less than 0.05 were considered to indicate statistically significant differences.

## Results

After subculture for 3 days, cells were cultured for 21 days in the presence of AHZ ($10^{-7}$-$10^{-5}$ M). During culture, the dishes were changed with the medium containing AHZ at the 3-day intervals. The alteration of alkaline phosphatase activity in the cells is shown in Fig. 1. When the cells were cultured for 12 days in the medium without AHZ (control), the cellular alkaline phosphatase activity increased about 5-fold in comparison with the value obtained at the zero time. In the presence of AHZ ($10^{-7}$, $10^{-6}$ and $10^{-5}$ M), the enzyme activity in the cells were increased progressively during 6 days of culture. Further increase was not seen in the cells treated with $10^{-7}$ and $10^{-6}$ M AHZ for 12 and 21 days. With $10^{-5}$ M AHZ, the cellular enzyme activity was further increased by the 12-day culture, and then it began to decrease with the longer culture. Meanwhile, protein concentration in the cells was significantly increased by the presence of AHZ ($10^{-7}$, $10^{-6}$ and $10^{-5}$ M) (Fig. 2). This increase was progressive during the culture with 21-day periods, although the dose-dependent effect of AHZ was not seen. The presence of AHZ ($10^{-7}$ to $10^{-5}$ M) did not cause a decrease of protein concentration in the medium with cell culture (data not shown).

The effects of AHZ and zinc sulfate on alkaline phosphatase activity and protein concentration in osteoblastic cells were compared with the culture of 1, 3 and 12 days. The medium containing either vehicle, $10^{-6}$ M AHZ or $10^{-6}$ M zinc sulfate was changed with the 3-day intervals. At one day with the culture, alkaline phosphatase activity in the cells was significantly increased by the presence of zinc sulfate or AHZ, although the AHZ effect was more intensive than that of zinc sulfate. A great increase of the enzyme activity with AHZ treatment was also seen by the culture for 3 and 12 days (Fig. 3). Meanwhile, protein concentration in the cells was not significantly increased by the presence of $10^{-6}$ M zinc sulfate or $10^{-6}$ M AHZ with the 1-day culture (Fig. 4). With the 3- and 12-day culture, AHZ produced a significant increase in the cellular protein concentration, although zinc sulfate did not have an appreciable effect. Thus, AHZ had an intensive effect on osteoblastic cells in comparison with the effect of zinc sulfate.

The effect of cycloheximide on AHZ-increased alkaline phosphatase activity and protein concentration in

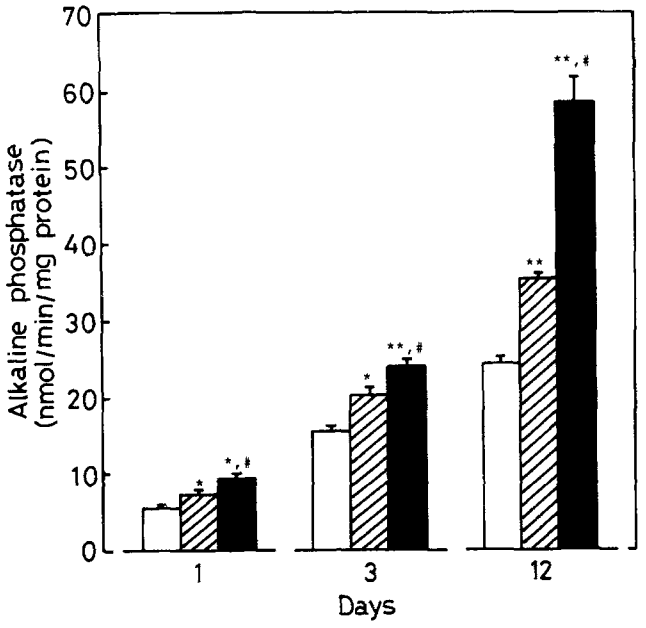


*Fig. 3.* Comparison with the effects of AHZ and zinc sulfate on alkaline phosphatase activity in osteoblastic MC3T3-E1 cells. Cells were cultured for 1, 3 and 12 days in medium containing either vehicle, AHZ ($10^{-6}$ M) or zinc sulfate ($10^{-6}$ M). Each value is the mean ± SEM of 6 dishes. * $P < 0.05$ and ** $P < 0.01$, as compared with the control value. # $P < 0.01$, as compared with the zinc sulfate value. □; Control, ▨; zinc sulfate, ■; AHZ.

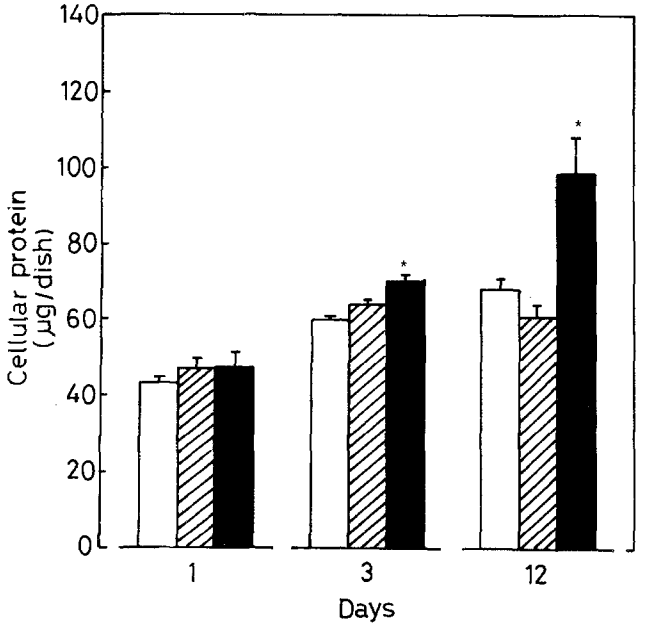


*Fig. 4.* Comparison with the effects of AHZ and zinc sulfate on protein concentration in osteoblastic MC3T3-E1 cells. Cells were cultured for 1, 3 and 12 days in medium containing either vehicle, AHZ ($10^{-6}$ M) or zinc sulfate ($10^{-6}$ M). Each value is the mean ± SEM of 6 dishes. * $P < 0.01$, as compared with the control value. □; Control, ▨; zinc sulfate, ■; AHZ.

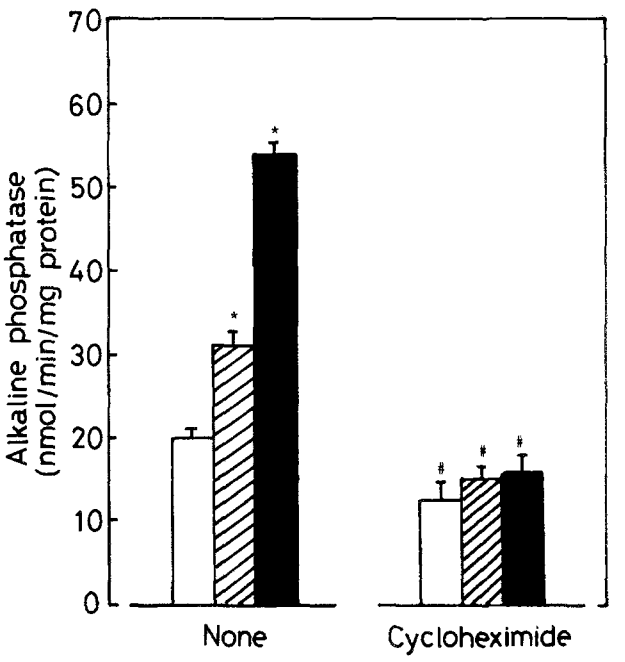


*Fig. 5.* Effect of cycloheximide on AHZ-increased alkaline phosphatase activity in osteoblastic MC3T3-E1 cells. Cells were cultured for 3 days in medium containing either vehicle, AHZ ($10^{-6}$ M) or zinc sulfate ($10^{-6}$ M), and then the medium was changed, and the cells were further cultured for 3 days in the presence of either cycloheximide ($10^{-6}$ M), cycloheximide ($10^{-6}$ M) plus AHZ ($10^{-6}$ M), or cycloheximide ($10^{-6}$ M) plus zinc sulfate ($10^{-6}$ M). Each value is the mean ± SEM of 6 dishes. * $P < 0.01$, as compared with the control value. # $P < 0.01$, as compared with the each value without cycloheximide. □; Control, ▨; zinc sulfate, ■; AHZ.

osteoblastic cells was examined. Cells were cultured for 3 days in medium containing $10^{-6}$ M AHZ or $10^{-6}$ M zinc sulfate, and then the medium was changed and the cells were further cultured for 3 days in the presence of $10^{-6}$ M cycloheximide plus either $10^{-6}$ M AHZ or $10^{-6}$ M zinc sulfate. The AHZ or zinc sulfate-induced increase in the cellular alkaline phosphatase activity was completely abolished by the presence of $10^{-6}$ M cycloheximide. Now, the presence of an inhibitor caused a significant decrease of the enzyme activity in the cells cultured without the zinc compounds (Fig. 5). Meanwhile, the effect of AHZ ($10^{-6}$ M) to increase the cellular protein concentration was completely prevented by the presence of $10^{-6}$ M cycloheximide (Fig. 6). Also, the presence of cycloheximide caused a significant decrease of protein concentration in the cells cultured in the absence of the zinc compounds.

When cells were cultured for 6 days in the medium containing either vehicle, AHZ ($10^{-6}$ M) or zinc sulfate ($10^{-6}$ M), the alteration of DNA content and cell number in the cells is shown in Table 1. The presence of AHZ

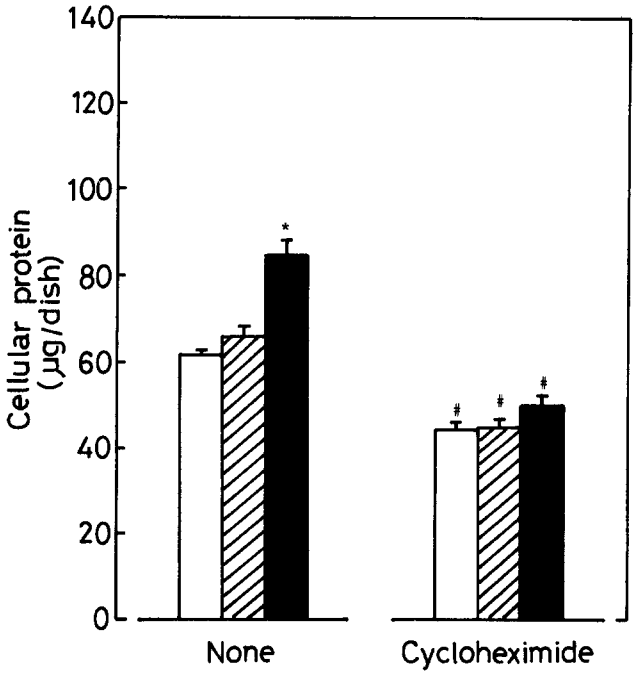


*Fig. 6.* Effect of cycloheximide on AHZ-increased protein concentration in osteoblastic MC3T3-E1 cells. Cells were cultured for 3 days in medium containing either vehicle, AHZ ($10^{-6}$ M) or zinc sulfate ($10^{-6}$ M), and then the medium was changed, and the cells were further cultured for 3 days in the presence of either cycloheximide ($10^{-6}$ M), cycloheximide ($10^{-6}$ M) plus AHZ ($10^{-6}$ M), or cycloheximide ($10^{-6}$ M) plus zinc sulfate ($10^{-6}$ M). Each value is the mean ± SEM of 6 dishes. * $P < 0.01$, as compared with the control value. # $P < 0.01$, as compared with the each value without cycloheximide. □; Control, ▨; zinc sulfate, ■; AHZ.

produced a significant increase of DNA content in the cells, although an appreciable elevation of cell number was not seen. Meanwhile, the presence of zinc sulfate did not have an effect on DNA content and cell number in osteoblastic cells. Thus, the effect of AHZ to increase protein concentration in the cells was not based on an alteration of cell number.

*Table 1.* Effect of AHZ or zinc sulfate on DNA content and cell number in osteoblastic MC3T3-E1 cells

| Treatment | DNA content (μg/dish) | Cell number ($\times 10^5$/dish) |
|---|---|---|
| Control | 5.73 ± 0.08 | 5.29 ± 0.19 |
| Zinc sulfate ($10^{-6}$ M) | 6.01 ± 0.28 | 5.30 ± 0.21 |
| AHZ ($10^{-6}$ M) | 7.46 ± 0.08** | 6.11 ± 0.33* |

Cells were cultured for 6 days in the medium containing either vehicle, zinc sulfate ($10^{-6}$ M) or AHZ ($10^{-6}$ M). Each value is the mean ± SEM of 6 dishes. * $P < 0.05$ and ** $P < 0.01$, as compared with the control value.

## Discussion

A new compound β-alanyl-L-histidinato zinc (AHZ), in which zinc is chelated to β-alanyl-L-histidine (L-carnosine), has a stimulatory effect on bone formation and calcification in weanling rats *in vivo* [7]. AHZ also has a direct stimulatory effect on bone formation in tissue culture *in vitro* [8]. The AHZ effect was more intensive than that of zinc sulfate *in vivo* [7] and *in vitro* [8]. More recently, it has been demonstrated that AHZ has a direct specific proliferative effect on osteoblastic cells *in vitro* and that this effect is dependent on protein synthesis [9]. Furthermore, the present investigation was undertaken to clarify the effect of AHZ on cell differentiation in osteoblastic MC3T3-E1 cells with the prolonged cultivation.

Alkaline phosphatase is known as a marker enzyme on the differentiation in osteoblastic cells [15, 16]. When osteoblastic cells were reached to subconfluent with pre-culture for 3 days, the cells were further cultured for 21 days in serum-free medium containing AHZ ($10^{-7}$ to $10^{-5}$ M) in the range of effective concentrations to stimulate bone formation in tissue culture system [8]. Between 6 and 12 days of culture, the presence of AHZ produced a progressive increase of alkaline phosphatase activity in osteoblastic cells with the time-dependency of culture, although this increase was weakened with the 21-day culture. Meanwhile, the cellular protein concentration was progressively increased during the 21-day culture in the presence of AHZ. Thus, a great increase of alkaline phosphatase in osteoblastic cells may be found in the proteins increased by the 6- and 12-day culture with AHZ. Presumably, it is assumed that AHZ may have a stimulatory effect on the differentiation in osteoblastic cells, and that this effect may be partly involved on an increase in the cellular protein concentration.

The effect of AHZ to increase alkaline phosphatase activity and protein concentration in osteoblastic cells was more intensive than that of zinc sulfate. This result further supports the view that the mode of AHZ action differs from that of zinc sulfate in osteoblastic cells. Presumably, AHZ may readily permeate into the cells as compared with zinc sulfate, and the metal chelated to dipeptide does not easily bind to cellular proteins which are inactive metallic action. The AHZ-induced increases of alkaline phosphatase activity and protein concentration in osteoblastic cells were completely abolished by the presence of cycloheximide, an inhibitor of protein synthesis at a translational process. This suggests that

the AHZ effect is dependent on a newly synthesized protein component. Meanwhile, the zinc-increased alkaline phosphatase activity was completely blocked by the presence of cycloheximide, although the metal did not have an appreciable effect on total protein concentration in osteoblastic cells. Alkaline phosphatase is a zinc-enzyme [17]. However, the present result suggests that the effect of zinc to increase the enzyme activity is based on the synthesis of the enzyme protein in osteoblastic cells. Zinc has been demonstrated to activate aminoacyl-tRNA synthetase and to increase newly synthesized protein components in bone tissue culture [5, 6]. Now, the culture with AHZ for 6 days produced a significant increase of DNA content and cell number in osteoblastic cells, while zinc had no effect. The present results suggest thatAHZ stimulates a transcriptional process in cellular nuclei. Previous study showed that AHZ-induced increases in DNA and proliferation of osteoblastic cells with a short-term cultivation (2 days) are abolished by the presence of an inhibitor of protein synthesis (cycloheximide) [9]. Presumably, AHZ stimulates the proliferation and differentiation, dependent on protein synthesis, in osteoblastic cells. The characterization of AHZ-induced protein components remains to be elucidated.

Now, despite of the prolonged cultivation with AHZ, osteoblastic cells maintained a great increase of alkaline phosphatase activity and protein concentration in the cells. Also, the presence of AHZ did not cause a significant decrease of osteoblastic cells with the 21-day cultivation (data not shown). Thus, AHZ may not have a toxic effect on osteoblastic cells. This may give an advantage for a pharmacologic role as a stimulator in osteoblastic cells.

In conclusion, it has been demonstrated that AHZ can increase alkaline phosphatase activity and protein concentration in osteoblastic cells with the prolonged cultivation, suggesting that AHZ has a stimulatory effect on the differentiation of cells in addition to the proliferative effect.

# References


1. Burch RE, Hahn HKJ, Sullivan JF: Newer aspects of the roles of zinc, manganase and copper in human nutrition. Clin Chim 21: 501–520, 1975
2. Hurley LS, Gowan J, Milhaud G: Calcium metabolism in manganese-deficient and zinc-deficient rats. Proc Soc Exp Biol Med 130: 856–860, 1969
3. Oner G, Bhaumick B, Bala EM: Effect of zinc deficiency on serum somatomedin levels and skeletal growth in young rats. Endocrinology 114: 1860–1863, 1984
4. Yamaguchi M, Yamaguchi R: Action of zinc on bone metabolism in rats: Increases in alkaline phosphatase activity and DNA content. Biochem Pharmacol 35: 773–777, 1986
5. Yamaguchi M, Oishi H, Suketa Y: Stimulatory effect of zinc on bone formation in tissue culture. Biochem Pharmacol 36: 4007–4012, 1987
6. Yamaguchi M, Oishi H, Suketa Y: Zinc stimulation of bone protein synthesis in tissue culture: Activation of aminoacyl-tRNA synthetase. Biochem Pharmacol 37: 4075–4080, 1988
7. Yamaguchi M, Oishi K: A new zinc compound, β-alanyl-L-histidinato zinc, stimulates bone growth in weanling rats. Res Exp Med 190: 105–110, 1990
8. Yamaguchi M, Miwa H: Stimulatory effect of β-alanyl-L-histidinato zinc on bone formation in tissue culture. Pharmacology 42: 230–240, 1991
9. Hashizume M, Yamaguchi M: Stimulatory effect of β-alanyl-L-histidinato zinc on cell proliferation is dependent on protein synthesis in osteoblastic MC3T3-E1 cells. Mol Cell Biochem, 122: 59–64, 1993
10. Sudo H, Kodama H, Amagai Y, Yamamoto S, Kasai S: *In vitro* differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 96: 191–198, 1988
11. Walter K, Schutt C: Acid and alkaline phosphatase in serum. In: Bergmeyer HU (ed.) Methods in enzymatic analysis. Verlag Chemie Weinheim Academic Press, New York, 1974, vol 1–2, p 856–860
12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275, 1951
13. Flanagan B, Nichols G Jr: Metabolic studies of bone *in vitro*. IV: Collagen biosynthesis by surviving bone fragments *in vitro*. J Biol Chem 237: 3786–3792, 1962
14. Ceriotti G: Determination of nuclei acids in animal tissues. J Biol Chem 214: 39–77, 1955
15. Bernier SM, Goltzman D: Effect of protein and steroidal osteotropic agents on differentiation and epidermal growth factor-mediated growth of the CFK1 osseous cell line. J Cell Physiol 152: 317–327, 1992
16. Stein GS, Lian JB, Owen TA: Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. FASEB J 4: 3111–3123, 1990
17. Parisi AF, Vallee BL: Zinc metalloenzyme: characteristics and significance in biology and medicine. Am J Clin Nutr 22: 1222–1230, 1969