Pergamon

Biochemical Pharmacology, Vol. 48, No. 6, pp. 1225–1230, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00292-4

INHIBITORY EFFECT OF ZINC COMPOUNDS ON OSTEOCLAST-LIKE CELL FORMATION IN MOUSE MARROW CULTURES

SEIKO KISHI and MASAYOSHI YAMAGUCHI*

Laboratory of Metabolism and Endocrinology, Graduate School of Nutritional Sciences, University of Shizuoka, Shizuoka City 422, Japan

(*Received* 7 *February* 1994; *accepted* 12 *May* 1994)

Abstract—The effect of zinc compounds on osteoclast-like cell formation in mouse marrow culture *in vitro* was investigated. The bone marrow cells were cultured for 7 days in α-minimal essential medium containing a well-known bone resorbing agent [1,25-dihydroxyvitamin D_3, parathyroid hormone (1–34), interleukin-1α or prostaglandin E_2]. Osteoclast-like cell formation was estimated by staining for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts. The presence of 1,25-dihydroxyvitamin D_3 (10^{-8} M), parathyroid hormone (10^{-8} M), interleukin-1α (50 U/mL) or prostaglandin E_2 (10^{-6} M) induced a remarkable increase in osteoclast-like multinucleated cells. These increases were inhibited by the presence of zinc sulfate or zinc-chelating dipeptide (β-alanyl-L-histidinato zinc; AHZ) in the concentration range of 10^{-8} to 10^{-5} M. The inhibitory effect of AHZ (10^{-8} and 10^{-7} M) was more intensive than that of zinc sulfate. Furthermore, the presence of Ni^{2+}, Cu^{2+}, Mn^{2+} or Co^{2+} (10^{-7} and 10^{-6} M) did not have an effect on parathyroid hormone (10^{-8} M)-induced osteoclast-like cell formation. The present study clearly demonstrates that zinc compounds have a potent inhibitory effect on osteoclast-like cell formation in mouse marrow culture.

Key words: zinc; β-alanyl-L-histidinato zinc; osteoclast formation; bone metabolism; bone marrow cells

It is known that zinc 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]. In recent years, it has been demonstrated that zinc has a stimulatory effect on bone formation and mineralization *in vivo* and *in vitro*, and that bone protein synthesis is a necessary component of this response [4–6]. Thus, zinc plays a physiological role as an activator in the regulation of bone formation.

On the other hand, AHZ†, in which zinc is chelated to β-alanyl-L-histidine, is a new compound. AHZ can stimulate bone growth in weanling rats [7] and bone calcification in aged rats [8]. Moreover, AHZ directly stimulates bone formation in tissue culture using the calvaria from weanling rats [9], and the compound also has a direct anabolic effect on osteoblastic cells (MC3T3-E1) *in vitro* [10]. AHZ has a more potent effect than zinc sulfate on bone formation [7, 9, 10], suggesting a role in the treatment of osteoporosis.

The effect of zinc compounds on bone resorption, however, has not been fully clarified. Therefore, the present study was undertaken to clarify whether zinc sulfate and AHZ have an effect on bone resorption *in vitro*. We examined the effect of zinc compounds on osteoclast-like cell formation in a mouse marrow culture system. It was found that zinc compounds have an inhibitory effect on osteoclast-like cell formation induced by a well-known bone resorbing agent.

MATERIALS AND METHODS

Chemicals. α-MEM and penicillin-streptomycin solution (5000 U/mL penicillin; 5000 μg/mL streptomycin) were obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). Fetal bovine serum was obtained from Bioproducts Inc. (Walkersville, MD, U.S.A.). $1,25(OH)_2D_3$, PGE_2, estrogen and acetazolamide were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Synthetic human PTH (1–34) and synthetic [$Asu^{1,7}$]eel CT were supplied by the Asahi Chemical Industry Co., Ltd. (Tokyo, Japan). Recombinant human IL-1α (10^8 U/mg) was obtained from the Genzyme Corp. (Boston, MA, U.S.A.). Zinc sulfate and other chemicals were of reagent grade from Wako Pure Chemical Industries (Osaka, Japan). AHZ, which was supplied by the Zeria Pharmaceutical Co. (Tokyo, Japan), was dissolved in hydrochloric acid and neutralized with sodium hydroxide. Other chemicals were dissolved in glass-distilled water.

Animals. Male mice (Ddy strain; 6 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 1.1% calcium, 1.1% phosphorus

* Corresponding author: Masayoshi Yamaguchi, Ph.D., Laboratory of Metabolism and Endocrinology, Graduate School of Nutritional Sciences, University of Shizuoka, 52–1 Yada, Shizuoka City 422, Japan. Tel (FAX). 54-264-5580.

† Abbreviations: AHZ, β-alanyl-L-histidinato zinc; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; PTH, parathyroid hormone; IL-1α, interleukin-1α; PGE_2, prostaglandin E_2; TRACP, tartrate-resistant acid phosphatase; α-MEM, α-minimal essential medium; CT, calcitonin; and MNCs, multinucleated cells.

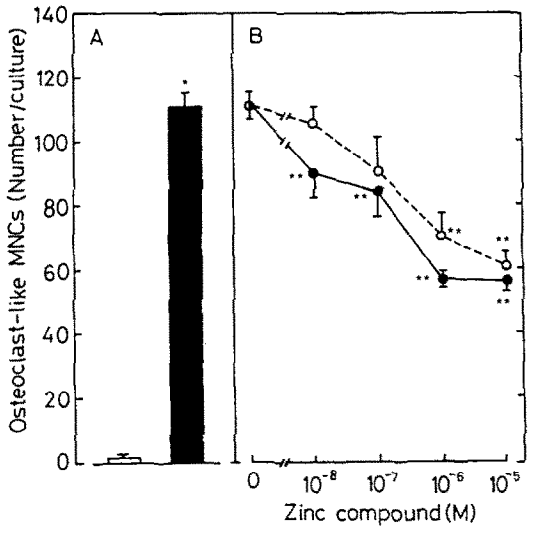

Fig. 1. Effect of zinc compounds on $1,25(OH)_2D_3$-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle (control), $1,25(OH)_2D_3$ (10^{-8} M), $1,25(OH)_2D_3$ plus zinc sulfate (10^{-8} to 10^{-5} M) or $1,25(OH)_2D_3$ plus AHZ (10^{-8} to 10^{-5} M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. The results are expressed as the means ± SEM of six cultures. Key: (*) $P < 0.01$, compared with the control group; and (**) $P < 0.01$, compared with the value for $1,25(OH)_2D_3$ alone. (A) control (□) or $1,25(OH)_2D_3$ (■); (B) $1,25(OH)_2D_3$ plus zinc (○) or $1,25(OH)_2D_3$ plus AHZ (●).

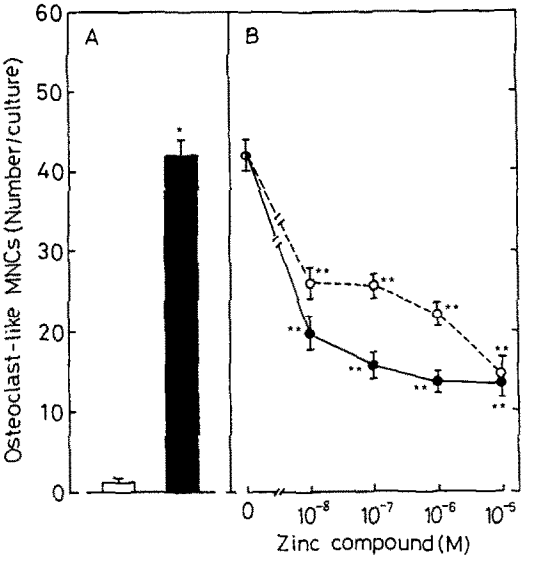

Fig. 2. Effect of zinc compounds on PTH-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle (control), PTH (10^{-8} M), PTH plus zinc sulfate (10^{-8} to 10^{-5} M) or PTH plus AHZ (10^{-8} to 10^{-5} M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. The results are expressed as the means ± SEM of six cultures. Key: (*) $P < 0.01$, compared with the control group; and (**) $P < 0.01$, compared with the value for PTH alone. (A) control (□) or PTH (■); (B) PTH plus zinc (○) or PTH plus AHZ (●).

and 0.012% zinc, and distilled water. The rats were killed by exsanguination.

Marrow cultures. Bone marrow cells were isolated from mice, as described elsewhere [11]. Briefly, bone ends of the femur were cut off, and the marrow cavity was flushed with 1 mL of α-MEM. The marrow cells were washed with α-MEM and cultured in the same medium containing 10% heat-inactivated fetal bovine serum at 1.0×10^7 cells/mL in 24-well plates (0.5 mL/well) in a water-saturated atmosphere containing 5% CO_2 and 95% air at 37°. The cells were cultured for 3 days; then 0.2 mL of the old medium was replaced with fresh medium and the cultures were maintained for an additional 4 days. Various concentrations of zinc sulfate or AHZ were added to the culture medium containing either vehicle, $1,25(OH)_2D_3$ (10^{-8} M), PTH (10^{-8} M), IL-1α (50 U/mL) or PGE_2 (10^{-6} M) with an effective concentration at the beginning of the cultures and at each time the medium was changed. In the separate experiments, the respective media contained either CT, 17β-estradiol or acetazolamide.

Enzyme histochemistry. After being cultured for 7 days, cells adherent to the 24-well plates were stained for TRACP, a marker enzyme of osteoclasts [12]. Briefly, cells were washed with Hanks' balanced salt solution and fixed with 10% neutralized formalin-phosphate (pH 7.2) for 10 min. After the culture dishes were dried, TRACP-staining was applied according to the method of Burstone [13]. The fixed cells were incubated for 12 min at room temperature (25°) in an acetate buffer (pH 5.0) containing naphthol AS-MX phosphate (Sigma) as a substrate, and red violet LB salt (Sigma) as a stain for the reaction product, in the presence of 10 mM sodium tartrate [14]. TRACP-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclast-like cells.

Pit formation assay. The pit formation assay was performed according to the method of Takada *et al.* [15] with some modifications. Briefly, transverse slices of dentine (150 μm in thickness) were prepared using a low-speed diamond saw (Leitz, Wetzlar, Germany). Each slice was ground to 100 μm in thickness and sterilized in 70% ethanol overnight. For this assay, whole bone marrow cells were cultured. After incubation for 7 days in the presence of $1,25(OH)_2D_3$ (10^{-8} M), the slices were examined for TRACP staining. After counting TRACP-positive MNCs on a slice, the slice was subjected to ultrasonication to remove attached cells and subsequently stained with toluidine blue (0.1%, w/v). The number of pits formed on the slices was determined using a light microscope.

Statistical analysis. All results are expressed as means ± SEM. The statistical significance of the control and the experimental group was analyzed by Student's *t*-test. P values of less than 0.05

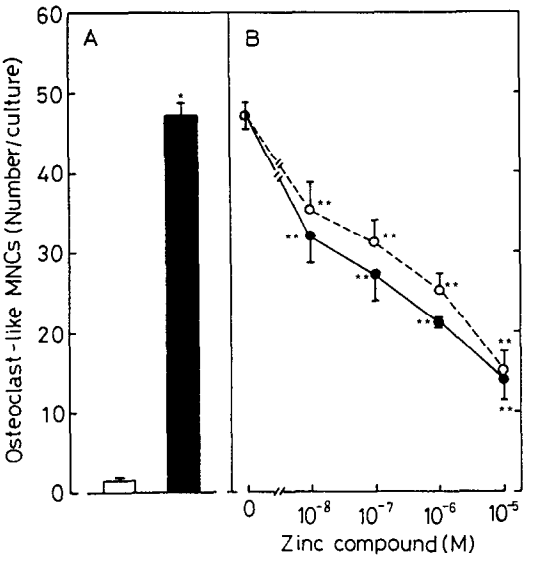

Fig. 3. Effect of zinc compounds on IL-1α-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle (control), IL-1α (50 U/mL), IL-1α plus zinc (10^{-8} to 10^{-5} M) or IL-1α plus AHZ (10^{-8} to 10^{-5} M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. The results are expressed as the means ± SEM of six cultures. Key: (*) $P < 0.01$, compared with control group; and (**) $P < 0.01$, compared with the value for IL-1α alone. (A) control (□) or IL-1α (■); (B) IL-1α plus zinc (○) or IL-1α plus AHZ (●).

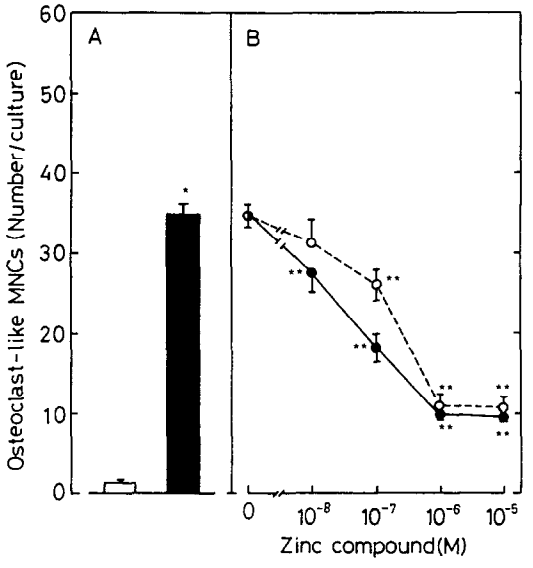

Fig. 4. Effect of zinc compounds on PGE_2-induced osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in medium containing either vehicle (control), PGE_2 (10^{-6} M), PGE_2 plus zinc (10^{-8} to 10^{-5} M) or PGE_2 plus AHZ (10^{-8} to 10^{-5} M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. The results are expressed as the means ± SEM of six cultures. Key: (*) $P < 0.01$, compared with the control group; and (**) $P < 0.01$, compared with the value for PGE_2 alone. (A) control (□) or PGE_2 (■); (B) PGE_2 plus zinc (○) or PGE_2 plus AHZ (●).

were considered to indicate statistically significant differences.

RESULTS

The effect of zinc compounds on the $1,25(OH)_2D_3$-induced osteoclast-like MNC formation in the mouse marrow culture system is shown in Fig. 1. When mouse marrow cells were cultured for 7 days with an effective concentration (10^{-8} M) of $1,25(OH)_2D_3$, the number of TRACP-positive MNCs was increased markedly (Fig. 1A). No TRACP-positive MNCs were formed in the control culture without $1,25(OH)_2D_3$ at any incubation time. The presence of AHZ (10^{-8} to 10^{-5} M) in the culture medium caused a significant decrease of the number of TRACP-positive MNCs increased by $1,25(OH)_2D_3$ (10^{-8} M) (Fig. 1B). In the presence of zinc sulfate, the decrease was seen at concentrations of 10^{-6} and 10^{-5} M. Zinc compounds in the range of 10^{-8} to 10^{-5} M did not have an inhibitory effect on the proliferation of marrow cells, which were cultured in medium with and without $1,25(OH)_2D_3$ (10^{-8} M) (data not shown).

The number of TRACP-positive MNCs was increased significantly by the presence of PTH (10^{-8} M) (Fig. 2A). This increase was inhibited by the essence of AHZ or zinc sulfate in the range of 10^{-8} to 10^{-5} M (Fig. 2B). The inhibitory effect of AHZ, however, was greater than that of zinc sulfate in the range of 10^{-8} to 10^{-6} M. At 10^{-5} M, the inhibitory effects of the zinc compounds were equal. Also, the presence of 10^{-8} to 10^{-5} M AHZ or zinc sulfate significantly blocked the IL-1α (50 U/mL)-induced increase in the number of TRACP-positive MNCs (Fig. 3). Moreover, the PGE_2 (10^{-6} M)-induced osteoclast-like MNC formation was inhibited by the presence of AHZ (10^{-8} to 10^{-5} M). The inhibitory effect of zinc sulfate was seen at 10^{-7} to 10^{-5} M (Fig. 4).

In another experiment, mouse marrow cells were cultured for 3 days in medium containing either vehicle, $1,25(OH)_2D_3$ (10^{-8} M), PTH (10^{-8} M), IL-1α (50 U/mL) or PGE_2 (10^{-6} M); then AHZ (10^{-6} M) or zinc sulfate (10^{-6} M) was added into the culture medium containing each bone-resorbing agent, and the cells were further incubated for 4 days. In this case, the presence of AHZ or zinc sulfate caused a significant inhibition of osteoclast-like MNC formation induced by all bone-resorbing agents (Table 1). This result indicates that zinc compounds can reveal an inhibitory effect at the later stage of osteoclast-like MNC formation with differentiation of marrow cells.

The effects of AHZ and other agents on the $1,25(OH)_2D_3$- or PTH-induced osteoclast-like cell formation in mouse marrow culture were compared, and the result is shown in Table 2. Mouse marrow

Table 1. Effect of zinc compounds on the bone-resorbing agent-induced osteoclast-like cell formation in mouse marrow culture

Treatment	Osteoclast-like MNCs (number/culture)			
	$1,25(OH)_2D_3$	PTH	IL-1α	PGE_2
Control	115.4 ± 6.2	44.3 ± 2.4	47.6 ± 1.5	34.6 ± 1.5
AHZ (10^{-6} M)	63.0 ± 3.1*	34.0 ± 2.3†	34.2 ± 2.8†	27.9 ± 3.0†
$ZnSO_4$ (10^{-6} M)	79.2 ± 5.9*	32.8 ± 3.8†	31.3 ± 2.8†	28.0 ± 2.9†

Mouse marrow cells were cultured for 7 days in medium containing either vehicle (control), $1,25(OH)_2D_3$ (10^{-8} M), PTH (10^{-8} M), IL-1α (50 U/mL) or PGE_2 (10^{-6} M). After 3 days of culture, the medium was changed, then AHZ or zinc sulfate was added in the culture medium containing each bone-resorbing agent, and the cells were cultured for an additional 4 days. Results are the means ± SEM of six cultures.

*,† Significantly different from the control value: *$P < 0.01$, and †$P < 0.05$.

Table 2. Comparison of the effects of AHZ and other agents on $1,25(OH)_2D_3$- or PTH-induced osteoclast-like cell formation in mouse marrow culture

Treatment	Osteoclast-like MNCs (number/culture)	
	$1,25(OH)_2D_3$	PTH
Control	109.8 ± 5.7	42.0 ± 2.0
AHZ 10^{-6} M	56.7 ± 2.8*	14.3 ± 1.2*
10^{-5} M	56.3 ± 4.4*	15.8 ± 1.1*
Calcitonin 3×10^{-10} M	43.8 ± 3.8*	22.7 ± 4.2*
3×10^{-9} M	34.6 ± 4.2*	12.2 ± 1.6*
17β-Estradiol 10^{-8} M	47.0 ± 5.1*	7.3 ± 1.1*
10^{-7} M	28.7 ± 1.1*	7.0 ± 0.7*
Acetazolamide 10^{-5} M	27.5 ± 1.2*	21.6 ± 2.4*
10^{-4} M	22.4 ± 2.1*	12.4 ± 1.5*

Mouse marrow cells were cultured for 7 days in medium containing either vehicle (control), $1,25(OH)_2D_3$ (10^{-8} M), PTH (10^{-8} M), or each bone-resorbing agent plus AHZ or other agents. Results are the means ± SEM of six cultures.

* $P < 0.01$, compared with the control value of $1,25(OH)_2D_3$ or PTH alone.

Table 3. Effects of various metals on $1,25(OH)_2D_3$- or PTH-induced osteoclast-like cell formation in mouse marrow culture

Treatment		Osteoclast-like MNCs (number/culture)	
		$1,25(OH)_2D_3$	PTH
Control		101.0 ± 6.8	43.1 ± 2.2
$ZnSO_4$	10^{-7} M	70.0 ± 6.0*	25.3 ± 1.9*
	10^{-6} M	64.0 ± 9.3*	22.3 ± 1.3*
$NiSO_4$	10^{-7} M	64.4 ± 2.8*	49.8 ± 4.7
	10^{-6} M	62.4 ± 2.2*	45.0 ± 3.5
$CuSO_4$	10^{-7} M	73.2 ± 3.6*	46.2 ± 2.8
	10^{-6} M	67.8 ± 3.3*	49.3 ± 0.7
$MnSO_4$	10^{-7} M	80.8 ± 2.9†	34.8 ± 2.3
	10^{-6} M	70.4 ± 3.2*	35.0 ± 3.8
$CoSO_4$	10^{-7} M	92.4 ± 2.1	38.0 ± 5.0
	10^{-6} M	86.4 ± 2.5	37.0 ± 4.5

Mouse marrow cells were cultured for 7 days in medium containing either vehicle (control), $1,25(OH)_2D_3$ (10^{-8} M), PTH (10^{-8} M), $1,25(OH)_2D_3$ plus each metal (10^{-7} and 10^{-6} M) or PTH plus each metal. Results are the means ± SEM of six cultures.

*,† Significantly different from the control value of $1,25(OH)_2D_3$ or PTH alone: *$P < 0.01$, and †$P < 0.05$.

cells were cultured for 7 days in medium containing either vehicle, AHZ (10^{-6} and 10^{-5} M), calcitonin (3×10^{-10} and 3×10^{-9} M), 17β-estradiol (10^{-8} and 10^{-7} M) or acetazolamide (10^{-5} and 10^{-4} M) in the presence of either $1,25(OH)_2D_3$ (10^{-8} M) or PTH (10^{-8} M). The presence of calcitonin, 17β-estradiol or acetazolamide caused a significant inhibition of osteoclast-like MNC formation induced by $1,25(OH)_2D_3$ or PTH. The inhibitory effect of AHZ was equal in comparison with that of other agents.

Moreover, the effects of various essential trace metals on the $1,25(OH)_2D_3$- or PTH-induced osteoclast-like cell formation in mouse marrow culture are shown in Table 3. In the presence of $1,25(OH)_2D_3$ (10^{-8} M), Zn^{2+}, Ni^{2+}, Cu^{2+} and Mn^{2+} at concentrations of 10^{-7} and 10^{-6} M significantly inhibited osteoclast-like cell formation in mouse marrow culture, whereas Co^{2+} (10^{-7} and 10^{-6} M) did not have an inhibitory effect. Meanwhile, in the presence of PTH (10^{-8} M), Zn^{2+} (10^{-7} and 10^{-6} M) clearly inhibited osteoclast-like cell formation in mouse marrow culture, although Ni^{2+}, Cu^{2+}, Mn^{2+} and Co^{2+} (10^{-7} and 10^{-6} M) had no effect. Thus, Zn^{2+} had a unique effect on osteoclast-like cell formation in mouse marrow cells.

We also cultured whole bone marrow cells on a dentine slice and examined the effect of zinc compounds on the number of resorption pits formed over 7 days. Zinc sulfate (10^{-6} M) or AHZ (10^{-6} M) clearly inhibited the $1,25(OH)_2D_3$ (10^{-8} M)-induced increase in the number of pits formed on a dentine slice; the number decreased from 82 ± 10 to 41 ± 7 or 29 ± 6 (mean ± SEM for six slices), respectively. The result supported the view that zinc compounds can inhibit the formation of osteoclasts from bone marrow cells.

DISCUSSION

AHZ, in which zinc is chelated to a dipeptide (β-alanyl-L-histidine), has a stimulatory effect on the proliferation and differentiation of osteoblastic cells *in vitro* [16, 17]. This zinc-chelating dipeptide can reveal a more intensive effect than zinc sulfate in osteoblastic bone formation [9, 16, 17]. Whether AHZ can influence the function of osteoclasts, however, has not been clarified thus far. The present study was undertaken to determine the effect of AHZ on the formation of osteoclast-like TRACP-positive MNCs from mouse marrow cells. It is well established that many bone-resorbing agents can stimulate the formation (differentiation) of osteoclasts from marrow cells [11, 18–21].

The presence of an effective concentration of $1,25(OH)_2D_3$, PTH, IL-1 α or PGE_2, potent bone-resorbing agents, clearly stimulated osteoclast-like cell formation in mouse marrow culture. The formation of osteoclast-like cells induced by these agents was inhibited markedly by the presence of zinc sulfate or AHZ. Zinc compounds did not have an inhibitory effect on the proliferation of marrow cells (data not shown), indicating that the compounds do not exhibit cytotoxicity toward marrow cells. The inhibitory effect of these zinc compounds was equal in comparison with the effect of other anti-bone-resorbing agents (calcitonin, 17β-estradiol and acetazolamide) on osteoclast-like cell formation in mouse marrow culture. Thus, it was found that zinc compounds have a potent inhibitory effect on osteoclast-like cell formation in mouse marrow culture.

The inhibitory effect of AHZ on osteoclast-like cell formation in mouse marrow culture seemed to be more potent than that of zinc sulfate. AHZ is a new compound in which zinc is chelated to β-alanyl-L-histidine. Now, β-alanyl-L-histidine (10^{-6} and 10^{-5} M) itself did not cause an appreciable effect on osteoclast-like cell formation in mouse marrow culture. Accordingly, the inhibitory effect of AHZ on osteoclast-like cell formation may require the chemical form of a zinc-chelating dipeptide. AHZ can easily enter bone cells (osteoblasts) in comparison with zinc sulfate [9, 10], and the zinc in AHZ may be partly released from the chelate. Zinc ion may play a role in the demonstration of the inhibitory effect of AHZ, since zinc sulfate had a significant inhibitory effect on osteoclast-like cell formation. At present, the cellular mechanism by which zinc compounds inhibit the formation of osteoclast-like cells induced by bone-resorbing agents in mouse culture is unknown. Since both AHZ and zinc sulfate have a stimulatory effect on protein synthesis in osteoblastic cells [6, 16, 17], the action of zinc compounds to stimulate protein synthesis may be somewhat involved in the differentiation of bone marrow cells. This remains to be elucidated.

Of various essential trace metals, Ni^{2+}, Cu^{2+}, Mn^{2+} and Co^{2+} did not have an inhibitory effect on PTH-induced osteoclast-like cell formation in mouse marrow culture, although Ni^{2+}, Cu^{2+} and Mn^{2+}, but not Co^{2+}, had an appreciable effect in inhibiting $1,25(OH)_2D_3$-induced osteoclast-like cell formation. The metals used, except for Zn^{2+}, did not have a potent inhibitory effect on osteoclast-like cell formation. Among various essential trace metals, zinc may be unique in the inhibition of osteoclast-like cell formation in mouse marrow culture.

Zinc compounds revealed an inhibitory effect at the later stage of differentiation of marrow cells. This observation suggests the possibility that zinc compounds can inhibit at least bone-resorbing activity of mature osteoclasts formed from marrow cells. It is possible that zinc is an inhibitor of the osteoclast ruffled membrane proton pump and, as such, could be having a direct effect on bone resorption, which is not addressed by the histochemical identification that we have employed. This, however, remains to be elucidated.

The oral administration of AHZ (10–100 mg/kg/day) for 3 months completely prevented bone loss in ovariectomized rats [20]. The inhibitory effect of AHZ on the formation of osteoclast-like cells may play a role in the prevention by AHZ of bone loss, although AHZ also has a potent stimulatory effect on osteoblastic bone formation *in vivo* [7, 8] and *in vitro* [9, 15, 16]. Zinc compounds may have a therapeutic role in osteoporosis.

REFERENCES

1. Burch RE, Hahn HKJ and Sullivan JF, Newer aspects of the roles of zinc, manganese, and copper in human nutrition. *Clin Chem* **21**: 501–520, 1975.
2. Hurley LS, Gowan J and Milhaoud G, Calcium metabolism in manganese-deficient and zinc-deficient rats. *Proc Soc Exp Biol Med* **130**: 856–860, 1969.
3. Oner G, Bhaumick B and Bala RM, Effect of zinc deficiency on serum somatomedin levels and skeletal growth in young rats. *Endocrinology* **114**: 1860–1863, 1984.
4. Yamaguchi M and 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 and Suketa Y, Stimulatory effect of zinc on bone formation in tissue culture. *Biochem Pharmacol* **36**: 4007–4012, 1987.
6. Yamaguchi M, Oishi H and 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 and Ozaki K, A new zinc compound, β-alanyl-L-histidinato zinc, stimulates bone growth in weanling rats. *Res Exp Med (Berl)* **190**: 105–110, 1990.
8. Yamaguchi M and Ozaki K, Effect of the new zinc compound beta-alanyl-*L*-histidinato zinc on bone metabolism in elderly rats. *Pharmacology* **41**: 345–349, 1990.
9. Yamaguchi M and Miwa H, Stimulatory effect of beta-alanyl-*L*-histidinato zinc on bone formation in tissue culture. *Pharmacology* **42**: 230–240, 1991.
10. Yamaguchi M and Ohtaki J, Effect of beta-alanyl-L-histidinato zinc on osteoblastic MC3T3-E1 cells: Increases in alkaline phosphatase and proliferation. *Pharmacology* **43**: 225–232, 1991.
11. Takahashi N, Yamana H, Yoshiki S, Roodman GD, Mundy GR, Jones SJ, Boyde A and Suda T, Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. *Endocrinology* **122**: 1373–1382, 1988.
12. Mundy GR and Roodman GD, Osteoclast ontogeny and function. In: *Bone and Mineral Research* (Ed. Peck

WA), Annual 5, pp. 209–279. Elsevier Science Publishers, Amsterdam, 1987.
13. Burstone MS, Histochemical demonstration of acid phosphatase with naphthol AS-phosphate. *J Natl Cancer Inst* **21**: 523–539, 1958.
14. Minkin C, Bone acid phosphatase: Tartrate-resistant acid phosphatase as a marker osteoclast function. *Calcif Tissue Int* **34**: 285–290, 1982.
15. Takada Y, Kusuda M, Hiura K, Sato T, Mochizuki H, Nagao Y, Tomura M, Yahiro M, Hakeda Y, Kawashima H and Kumegawa M, A simple method to assess osteoclast-mediated bone resorption using unfractionated bone cells. *Bone Miner* **17**: 347–359, 1992.
16. Hashizume M and 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.
17. Hashizume M and Yamaguchi M, Effect of β-alanyl-L-histidinato zinc on differentiation of osteoblastic MC3T3-E1 cells: Increases in alkaline phosphatase activity and protein concentration. *Mol Cell Biochem* **131**: 19–24, 1994.
18. Kurihara N, Suda T, Miura Y, Nakauchi H, Kodama H, Hiura K, Hakeda Y and Kumegawa M, Generation of osteoclasts from isolated hematopoietic progenitor cells. *Blood* **74**: 1295–1302, 1989.
19. Shinar DM and Rodan GA, Biphasic effects of transforming growth factor-β on the production of osteoclast-like cells in mouse bone marrow cultures: The role of prostaglandins in the generation of these cells. *Endocrinology* **126**: 3153–3158, 1990.
20. Takahashi N, Kukita T, MacDonald BR, Bird A, Mundy GR, McManus LM, Miller M, Boyde A, Jones SJ and Roodman GD, Osteoclast-like cells form in long-term human bone marrow but not in peripheral blood cultures. *J Clin Invest* **83**: 543–550, 1989.
21. Yamaguchi M and Kishi S, Prolonged administration of β-alanyl-L-histidinato zinc prevents bone loss in ovariectomized rats. *Jpn J Pharmacol* **63**: 203–207, 1993.