Preventive Effects of Zinc on Cadmium-Induced Inhibition of Alkaline Phosphatase Activity and Mineralization Activity in Osteoblast-Like Cells, MC3T3-E1

Yoshiharu SUZUKI,* Ikuo MORITA,** Yasuhiro YAMANE,* and Seiitsu MUROTA**

*Faculty of Pharmaceutical Science, Chiba University, * Yayoi-cho, Chiba, 260, Japan and Section of Physiological Chemistry, Institute of Stomatognathic Science, Tokyo Medical and Dental University, ** Yushima, Bunkyo, Tokyo, 113, Japan*

(Received August 12, 1988)

In order to determine the specific action of cadmium on bone metabolism, the effect of cadmium on alkaline phosphatase activity, a marker enzyme of osteoblasts, was compared with that of other divalent heavy metal ions, *i.e.*, zinc, manganese, lead, copper, nickel and mercury (10 μM each), using cloned osteoblast-like cells, MC3T3-E1. Cadmium had the strongest inhibitory effect on alkaline phosphatase activity of the cells among the metals tested. At the same dose, however, cadmium failed to inhibit cellular glucose-6-phosphatase and lactate dehydrogenase activities, suggesting that the inhibitory effect of cadmium on alkaline phosphatase was specific and was not dependent on cell injury. Cadmium treatment caused a significant decrease in cellular zinc level, but mercury treatment had no such effect at the dose inhibiting alkaline phosphatase activity. There was a good correlation between decrease of cellular zinc level and inhibition of alkaline phosphatase activity in cadmium-treated cells. Concomitant treatment of the cells with zinc prevented the cadmium-induced inhibition of alkaline phosphatase activity. However, this was not the case in the mercury-induced inhibition. Cadmium also inhibited the mineralization of osteoblasts. When 10 or 20 μM zinc was concomitantly added to the cultures, the inhibition of mineralization was prevented. These data suggest that the inhibitory effect of cadmium in osteoblasts may be closely related to its influence on the cellular zinc metabolism.

Keywords — cadmium; osteoblast; zinc; alkaline phosphatase; mineralization

Introduction

Chronic cadmium (Cd) intake causes hepatic, renal and bone disease, hypertension, anemia, *etc.*^{1–4)} As an example of the toxicity of Cd to bone metabolism, so-called Itai Itai disease associated with osteomalacia or osteoporosis is well known. In this disease, it has been considered that Cd acts secondarily on bone through abnormal calcium homeostasis due to renal failure caused by chronic Cd ingestion.⁵⁾ On the other hand, it has been reported that Cd is accumulated on osteoblasts in bone tissue⁶⁾ and can induce bone disorder without renal damage.⁷⁾ Moreover, Miyahara *et al.*^{8,9)} have reported that Cd can inhibit bone formation and stimulate bone resorption in a chick bone organ culture system. Thus, the direct action of Cd on bone tissue has recently been recognized. However, it is unclear what is the mechanism of the direct action of Cd on bone tissue. Bone metabolism is regulated by two kinds of cells, osteoblasts and osteoclasts,

which mediate bone formation and bone resorption, respectively. Recently Kodama *et al.*¹⁰⁾ have established an osteoblast-like cell line, MC3T3-E1, which maintains several of the features of osteoblasts *in vivo*, *i.e.*, high alkaline phosphatase (ALPase) activity, ability of bone-like collagen synthesis, mineralization and so on. Kumegawa *et al.*¹¹⁾ have characterized the responses of the cells to various kinds of hormones and autacoids. To clarify the mechanism of Cd intoxication in bone tissue, we examined the effects of Cd on bone formation using MC3T3-E1 cells. We will show that Zn can prevent the Cd-induced inhibition of ALPase activity and mineralization activity in MC3T3-E1 cells.

Materials and Methods

Cells and Culture Conditions — MC3T3-E1 cells established from newborn mouse calvaria by Kodama *et al.*¹⁰⁾ were cultivat-

ed in plastic dishes (Falcon, U.S.A.) with α -MEM (Gibco, U.S.A.) supplemented with 10% fetal bovine serum (Irvine Scientific, U.S.A.) under 5% CO₂ and 95% air at 37 °C. The cells were subcultivated every three days. The viability of cells treated with metals, tested by exclusion of erythrosin B, was greater than 95%.

Assay of Enzyme Activity — At the stationary phase of the cell growth, each metal ion solution was added to the medium to the final concentration of 10 μ M. After being cultivated for 24 h, cells were washed with ice-cold saline and scraped off with a rubber policeman. The scraped cells were sonicated and used for the assay of ALPase activity with *p*-nitrophenyl phosphate as a substrate (ALPase assay kit, Wako Pure Chem., Japan). One unit of enzyme activity was defined as the activity causing the release of 1 μ mol of *p*-nitrophenol per min. The effect of the Cd treatment was compared with that on other cellular enzymes, glucose-6-phosphatase (G-6-Pase) and lactate dehydrogenase (LDH). After the Cd (10 μ M) treatment for 24 h, cells were washed with ice-cold saline for ALPase or LDH assay and with ice-cold 0.25 M sucrose solution for G-6-Pase and samples were prepared as indicated above. LDH activity was assayed by reading the change of absorbance at 340 nm owing to oxidation of nicotinamide adenine dinucleotide (NADH) through the conversion from pyruvate to lactate (LDH assay kit, Wako Pure Chem., Japan). One unit of LDH activity was defined as the activity causing the conversion of 1 μ mol NADH per min. G-6-Pase activity was assayed by the modified method of Swanson.¹²⁾ The activity was expressed as nmol of Pi released from G-6-PNa₂ per 15 min at 37 °C. Data are given as the activities per 10⁶ cells.

Assay of Zn Content — After the treatment with the indicated concentration of Cd, cell homogenates were digested with HNO₃. Zn content was measured by atomic absorption spectrophotometry (Hitachi 170).

Effect of Zn Supplement on Cd-Induced Inhibition of Mineralization Activity in the Cells — At the stationary phase, cells were treated with Cd (2 μ M) or/and Zn (10 or 20 μ M) and cultivated for 9 d. The medium was

changed every 3 days and at the 7th day, 5 mM β -glycerophosphate was added. The degree of mineralization activity was compared with the control by the von Kossa method,¹³⁾ in which mainly calcium phosphate on the matrix is stained; this method is widely used for the detection of the mineral in various tissues.

Results

In order to clarify the specific action of cadmium on the function of osteoblasts, the effects of various heavy metal ions on ALPase activity, one of the most important markers of osteoblasts, were investigated (Table I). In the experiment, the concentration and incubation time of the metals tested were set at 10 μ M and 24 h, respectively, and these conditions had little effect on cell proliferation at the stationary phase (data not shown). Zn weakly stimulated ALPase activity of MC3T3-E1 cells. The other heavy metal ions tested showed a tendency to inhibit the enzyme activity. Among them, Cd had the most potent inhibitory effect (48% of the control).

To determine whether the decrease in ALPase activity caused by Cd treatment was specific, we measured G-6-Pase and LDH, which are commonly measured as markers of cell injury. As shown in Table II, no decrease in cellular G-6-Pase and LDH activities by Cd treatment were observed.

At the same time, the change in cellular Zn

TABLE I. The Effect of Various Divalent Heavy Metal Ions on ALPase Activity in MC3T3-E1 Cells

Treatment	ALPase activity
Control	39.1 $\pm$ 1.3
Zn	44.4 $\pm$ 1.9
Cu	33.4 $\pm$ 1.4
Ni	29.5 $\pm$ 0.2
Pb	26.4 $\pm$ 1.2
Mn	24.3 $\pm$ 0.7
Hg	22.6 $\pm$ 1.0
Cd	18.8 $\pm$ 1.0

At the stationary phase of cell growth, cells were treated with each metal ion (10 μ M) for 24 h and ALPase activity was assayed as described in Materials and Methods. Data represent means $\pm$ S.E. for three dishes.

TABLE II. The Effects of Cd on ALPase, G-6-Pase and LDH Activities in MC3T3-E1 Cells

Treatment	ALPase ^{a)}	G-6-Pase ^{b)}	LDH ^{a)}
Control	44.4 ± 1.5	22.1 ± 0.9	220.2 ± 12.7
Cd	26.4 ± 0.4 ^{c)}	20.5 ± 0.9	231.9 ± 4.6

At the stationary phase of cell growth, cells were treated with Cd (10 μ M) for 24 h. ALPase, G-6-Pase and LDH activities were assayed as described in Materials and Methods. Data represent means $\pm$ S.E. for three individual dishes. *a)* mU/10⁶ cells. *b)* nmol/15 min/10⁶ cells. *c)* Significantly different from the control ($p < 0.001$).

content caused by Cd treatment was measured, because it has been reported that ALPase activity is regulated by intracellular Zn and Mg in rat hepatoma cells.¹⁴⁾ Figure 1 shows the dose-effect relationships of Cd on Zn content and ALPase activity in MC3T3-E1 cells. Cd treatment caused a decrease in Zn content as well as in ALPase activity. The inhibition of ALPase activity seemed to be parallel with the decrease in cellular Zn content. On the other hand, mercury (Hg) treatment (10 μ M) had no effect on Zn content (10.0 $\pm$ 0.4 μ mol/10¹⁰ cells) at the dose inhibiting ALPase activity. These data suggest that Cd toxicity was related to the decrease in cellular Zn content. Therefore, we next examined if Zn treatment prevented Cd-induced inhibition of ALPase activity. As shown in Fig. 2, concomitant addition of Zn to the cultures prevented the decrease in ALPase activity of the cells. However, the preventive effect of Zn on ALPase activity was not found in the case of Hg treatment (Table III).

Mineralization was observed over the cell matrix during a 9-d culture with 5 mM β -glycerophosphate. The mineralization was abolished when the cells were treated with 2 μ M Cd for 9 d. When 10 or 20 μ M Zn was added to the cultures concomitantly, the inhibitory effect of Cd on mineralization was prevented in a dose-dependent manner (Fig. 3).

Discussion

In the Cd-induced intoxication of bone, it has been generally accepted that Cd acts directly on bone in part, and also secondarily on bone *via* abnormal calcium homeostasis due to chronic renal failure and so on. Recently isolated bone tissue was used to study the direct effects of Cd biochemically and histologically. Bawden and Hammarström demonstrated that Cd was localized mostly in osteoblasts in Cd-treated rat bone.⁶⁾ The evidence suggests that Cd can act directly on osteoblasts and inhibit bone forma-

Fig. 1. Dose-Effect Relationships for Cd on Zn Content and ALPase Activity in MC3T3-E1 Cells

Cells were treated with Cd (1, 3, 6 and 10 μ M) for 24 h. Data represent means of duplicate determination for two different dishes, and similar results were obtained from another experiment. A; Zn content (μ mol/10¹⁰ cells), B; ALPase activity (mU/10⁶ cells).

Fig. 2. The Effect of Cd Alone and Cd Plus Zn on ALPase Activity in MC3T3-E1 Cells

Cells were treated with Cd (10 μM) alone or with Cd (10 μM) and Zn (20 μM) for 24 h. Open bars and closed bars represent ALPase activity and Zn content in the cells, respectively. Data represent means ± S.E. for three individual dishes. Significantly different from the control, *a*) $p < 0.001$ and from only Cd treatment, *b*) $p < 0.01$, *c*) $p < 0.001$.

tion. In order to clarify the specific action of Cd on bone formation, we have studied the effects of Cd on ALPase activity and mineralization using isolated osteoblast-like cells, MC3T3-E1.

It has been recognized that ALPase is an important enzyme for the functions of osteoblasts.¹⁵⁾ As Cd has the most potent inhibitory effect on ALPase activity among various heavy metal ions tested (Table I), there is a possibility

TABLE III. The Effect of Concomitant Treatment with Zn and/or Hg on ALPase Activity of MC3T3-E1 Cells

Treatment	ALPase activity (mU/10 ⁶ cells)
Control	55.2 ± 1.8
Hg	34.1 ± 0.5 ^a
Zn	61.2 ± 1.8
Hg-Zn	36.3 ± 1.3 ^b

Cells were treated with Hg (10 μM) and/or Zn (20 μM) for 24 h. Data represent means ± S.E. for three individual dishes. Significantly different from the control (*a*) $p < 0.001$ and from Hg treatment (*b*) $p < 0.01$.

that Cd can induce bone disease through the inhibition of some functions of osteoblasts, such as ALPase, mineralization and so on. Because cellular G-6-Pase and LDH activities are not influenced by Cd treatment (Table II), the inhibitory effect of Cd on ALPase was specific and was not dependent on cell injury. There were some differences in the values of ALPase activity between the controls in our independent experiments. However, the phenotypes including ALPase activity in MC3T3-E1 cells have been recognized to be variable through a series of experiments. As the variance in each of our experiments was small, we concluded that our data

Fig. 3. The Effect of Cd Alone and Cd Plus Zn on Mineralization Activity of MC3T3-E1 Cells

Cells were treated with Cd (2 μM) or/and Zn (10 or 20 μM) for 9 d. β-Glycerophosphate (5 mM) was added only for the last 2 days. Mineralization was stained by the von Kossa method.

could be reliable.

Yamaguchi and Inamoto¹⁶⁾ reported that administration of Zn causes a significant elevation of ALPase activity in the rat femoral diaphysis. Recently, Sorimachi also showed that ALPase activity was activated by cellular Zn and Mg in cultured hepatoma cells.¹⁴⁾ Therefore, we examined whether the Cd intoxication was associated with Zn metabolism. Cd treatment caused a dose-dependent decrease in cellular Zn content (Fig. 1). The data suggest that Zn deficiency in the Cd-treated cells may be related to the inhibition of ALPase activity. The mechanism of the decrease in cellular Zn content by Cd is still unclear, but we assume that Cd inhibits the mobilization of Zn in MC3T3-E1 cells. In hepatocytes, Pattison and Cousins have shown the inhibitory effect of Cd on Zn incorporation.¹⁷⁾ As ALPase is a Zn-containing enzyme, the inhibition of ALPase activity by Cd treatment may be partly due to the disturbance of Zn metabolism in the cells. When Zn was added concomitantly with Cd, Cd-induced toxicity was prevented, but this is not the case in Hg-induced toxicity (Fig. 2, Table III). As Hg inhibited the ALPase activity of the cells without affecting cellular Zn content, the effect of Hg on ALPase activity may involve a different mechanism from the Cd-related one. When Zn was added to the culture after Cd treatment for 24 h, ALPase activity also increased in response to the recovery of cellular Zn content, as in the case of concomitant treatment (data not shown). So, in respect to Cd toxicity on osteoblasts, the involvement of Zn mobilization may be an important factor and it may modify the outbreak of the toxicity. On the other hand, it remains a possibility that Cd acts directly on the ALPase enzyme molecule. In *Escherichia coli*, it has been shown that Cd binds the apo-enzyme and Cd-ALPase has much lower phosphatase activity.¹⁸⁾ However, Zn has a much stronger affinity to the Zn binding site of ALPase compared to Cd.¹⁹⁾

Cd also abolished mineralization of the cell matrix (Fig. 3) as reported by Miyahara *et al.*²⁰⁾ The abolition of mineralization could not be caused only by the inhibition of ALPase activity. Miyahara *et al.*²⁰⁾ also claimed that the decrease in ALPase activity might not be related to the

inhibition of mineralization by Cd. However, high ALPase activity has been found histochemically in active osteoblasts and the enzyme has long been recognized to be very important for the process of bone mineralization.¹⁵⁾ Therefore, we consider that the disturbance of ALPase activity may be one of the mechanisms of inhibition of mineralization by Cd. The toxicity of Cd to mineralization was also prevented by concomitant addition of Zn to the cultures. These results suggest that the abolition of mineralization by Cd is related to the disturbance of Zn metabolism. It has been reported that a metallothionein (MT)-like protein is synthesized in bone tissue and in MC3T3-E1 cells.^{20,21)} Based on many experiments, it has been suggested that Zn can prevent Cd toxicity through the stimulation of MT synthesis.⁴⁾ In our experiments on simultaneous treatment with Cd and Zn, however, Cd bound to MT-like protein did not increase in a short-term or a long-term experiment (data not shown). So, the effects of Zn on Cd-induced inhibition of ALPase activity and mineralization activity may not involve a MT-related mechanism. Zn is an essential element and plays important roles in many organisms. Recently it has been indicated that Zn has a cooperative interaction with Ca on protein kinase C.²²⁾ Consequently, the decrease in cellular Zn content caused by the Cd-treatment may also lead to disruption of cell responses to external signals (hormones, *etc.*).

In conclusion, Cd has an inhibitory effect on ALPase activity and mineralization activity of osteoblast-like cells, MC3T3-E1, and the disturbance of Zn metabolism may be partly related to the Cd toxicity.

Acknowledgment We are grateful to Dr. H. Kodama for the generous gift of MC3T3-E1 cells.

References

- 1) R. L. Singhal, Z. Merali and P. D. Hrdina: Aspects of the biological toxicology of cadmium, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **35**, 75–80 (1976).
- 2) Y. Itokawa, T. Abe and S. Tanaka: Bone changes in experimental chronic cadmium poisoning, *Arch. Environ. Health*, **26**, 241–244 (1973).

- 3) F. N. Kotsonis and C. D. Klaassen: Toxicity and distribution of cadmium administered to rats at sublethal doses, *Toxicol. Appl. Pharmacol.*, **41**, 667—680 (1977).
- 4) B. Venugopal and T. D. Luckey: Cadmium "Metal Toxicity in Mammals 2," Plenum Press, New York, 1978, pp. 76—86.
- 5) S. E. Larsson and M. Piscator: Effect of cadmium on skeletal tissue in normal and calcium-deficient rats, *Metab. Bone Dis.*, **7**, 495—498 (1971).
- 6) J. W. Bawden and L. E. Hammarström: Distribution of cadmium in developing teeth and bone of young rats, *Scand. J. Dent. Res.*, **83**, 179—186 (1975).
- 7) S. Yoshiki, T. Yanagisawa, M. Kimura, N. Otaki and M. Suzuki: Bone and kidney lesions in experimental cadmium intoxication, *Arch. Environ. Health*, **30**, 559—562 (1975).
- 8) T. Miyahara, T. Kato, S. Nakagawa, H. Kozuka, T. Sakai and N. Takayanagi: Influence of poisonous metals on the bone metabolism. I. The effect of cadmium on the ossification of chick-embryo tibia in tissue culture, *Eisei Kagaku*, **24**, 36—42 (1978).
- 9) T. Miyahara, M. Miyahara, Y. Saito and H. Kozuka: Influence of poisonous metals on bone metabolism, *Toxicol. Appl. Pharmacol.*, **55**, 477—483 (1980).
- 10) H. Kodama, Y. Amagi, H. Sudo, S. Kasai and S. Yamamoto: Establishment of a clonal osteogenic cell line from new born mouse calvaria, *Jpn. J. Oral Biol.*, **23**, 899—901 (1982).
- 11) M. Kumegawa, E. Ikeda, S. Tanaka, T. Haneji, T. Yora, Y. Sakagishi, N. Minami and M. Hiramatsu: The effects of prostaglandin E₂, parathyroid hormone, 1, 25-dihydroxycholecalciferol and cyclic nucleotide analogs on alkaline phosphatase activity in osteoblastic cells, *Calcif. Tissue Int.*, **36**, 72—76 (1984).
- 12) M. A. Swanson: Glucose-6-phosphatase from liver, "Methods in Enzymology," Vol. 2, Academic Press, New York, 1955, pp. 541—543.
- 13) R. A. B. Drury, E. A. Wallington and R. Cameron: von Kossa Staining "Carleton's Histological Technique," 4th ed., Oxford University Press, London, 1973, p. 150.
- 14) K. Sorimachi: Activation of alkaline phosphatase with Mg and Zn in rat hepatoma cells, *J. Biol. Chem.*, **262**, 1535—1541 (1987).
- 15) A. J. Kahn, M. D. Fallon and S. L. Teitelbaum: Structure-function relationships in bone: an examination of events at the cellular level, "Bone and Mineral Research, Annual 2," ed. by A. P. Williams, Elsevier Science Publishers B. V., Amsterdam 1983, Chapter 3.
- 16) M. Yamaguchi and K. Inamoto: Differential effects of calcium-regulating hormones on bone metabolism in weanling rats orally administered zinc sulfate, *Metabolism*, **35**, 1044—1047 (1986).
- 17) S. E. Pattison and R. J. Cousins: Kinetics of zinc uptake and exchange by primary cultures of rat hepatocytes, *Am. J. Physiol.*, **250**, E677—E685 (1986).
- 18) C. Lazdunski, C. Petitclerc and M. Lazdunski: Structure-function relationships for some metalloalkaline phosphatases of *E. coli*, *Eur. J. Biochem.*, **8**, 510—517 (1969).
- 19) M. I. Harris and J. E. Coleman: The biosynthesis of apo- and metalloalkaline phosphatases of *Escherichia coli*, *J. Biol. Chem.*, **243**, 5063—5073 (1968).
- 20) T. Miyahara, H. Yamada, R. Ando, S. Nemoto, T. Kaji, M. Mori, H. Kozuka, N. Itoh and H. Sudo: The effects of cadmium on a clonal osteogenic cell, MC3T3-E1: Inhibition of calcification and induction of metallothionein-like protein by cadmium, *Toxicol. Lett.*, **32**, 19—27 (1986).
- 21) M. Kimura, N. Otaki, S. Yoshiki, M. Suzuki, N. Horiuchi and T. Suda: The isolation of metallothionein and its protective role in cadmium poisoning, *Arch. Biochem. Biophys.*, **165**, 340—348 (1974).
- 22) K. Murakami, M. K. Whiteley and A. Routtenberg: Regulation of protein kinase C activity by cooperative interaction of Zn and Ca, *J. Biol. Chem.*, **262**, 13902—13906 (1987).