Arch Toxicol (1993) 67: 352–357

Archives of
Toxicology

Comparative effect of cadmium on osteoblastic cells and osteoclastic cells

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Received 14 May 1992/Accepted 2 February 1993

Abstract. Cadmium(Cd) has been thought to disturb the bone metabolism directly. The mechanism for the bone lesion is unknown, however. To examine the effects of cadmium on bone metabolism, we compared its effects on osteoblasts and osteoclasts in vitro. We used an established cell line, MC3T3-E_1, as osteoblasts and tartrate resistant acid phosphatase (TRACP)-positive multi-nucleated cells (MNC) formed by a bone marrow culture system as osteoclasts. Alkaline phosphatase (ALP) activity was decreased by 10^{-7} M Cd and DNA content and hydroxyproline content of osteoblastic cells were decreased by 10^{-5} M Cd. Cadmium at 10^{-7} M inhibited the osteoclastic cell formation from mouse bone marrow in the presence of 10^{-8} M 1α,25$(OH)_2$ vitamin D_3. A 100-fold higher concentration of zinc(Zn) simultaneously added to the cadmium-containing medium prevented the toxicity of cadmium to osteoclastic cells as observed in the culture of osteoblastic cells. These results indicate that both bone formation and bone resorption are inhibited by cadmium. The responses of osteoclasts and osteoblasts to cadmium in this culture system were the same and the responses of cadmium-damaged osteoblasts and osteoclasts to zinc were also similar. These results suggest that another mechanism by which cadmium could cause bone damage should be considered in addition to the specific induction of osteoclastic cells by Cd.

Key words: Cadmium – Osteoclasts – Osteoblasts – Bone and bones

Introduction

The etiology of bone lesions, including osteomalacia and osteoporosis, caused by cadmium in human and experimental animals has been discussed. Two possible mechanisms by which Cd causes bone damage have been proposed. One possible mechanism is an indirect effect of Cd on bone metabolism. That is, bone mineral metabolism is disturbed because the activation of vitamin D in kidney is blocked by Cd (Feldman et al. 1973) or since Ca uptake by the intestine is inhibited by Cd (Yuhas et al. 1978). On the other hand, Kimura et al. (1974) have suggested that cadmium ions act directly on bone rather than by an indirect action through a functional disturbance of the kidney. Yoshiki et al. (1975) have also reported that before the occurrence of kidney damage, ingested Cd causes osteoporotic changes in rat bone tissue. We have reported that the bone strength of rats administered a low dose of Cd was decreased without kidney damage (Ogoshi et al. 1984). However, there are few reports on the mechanisms by which Cd causes bone lesions. We have reported that Cd inhibits the bone formation of cultured fetal rat calvaria (Iwami et al. 1990). Kaji et al. (1988) also reported that Cd inhibits the bone formation of cultured embryo chick tibia. On the other hand, there are several reports that Cd stimulates the bone resorption. Furuta (1978) showed that the number of osteoclastic cells was increased in femurs from Cd-exposed rats. Suzuki et al. (1989a) reported that Cd stimulates bone resorption of fetal mouse calvaria via a prostaglandin E_2-mediated mechanism.

In this study, we compared the effects of Cd on both osteoblasts and osteoclasts in culture to clarify the mechanism of Cd-induced bone damage. We used an established cell line, MC3T3-E_1, as osteoblasts, and TRACP-positive multi-nucleated cells formed by a bone marrow culture system as osteoclasts.

Materials and methods

Culture of osteoblastic cell line, MC3T3-E_1. A clonal osteogenic cell line, MC3T3-E_1, established by Kodama et al. (1981), was kindly provided by Dr. Kumegawa (Meikai University, School of Dentistry, Sakado, Saitama, Japan). The cells were cultured at 37° C in a fully humidified atmosphere of 5% CO_2 in air in 100-mm plastic dishes containing an α-modification of Eagle's Minimum Essential Medium (α-MEM) (Flow Laboratories, Irvine, Scotland) supplemented with 10%

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fetal bovine serum (FBS) (GIBCO Laboratories, Gland Island, NY) and 100 U/ml penicillin G. They were subcultured every 3 days using 0.001% actinase E (Kaken Kagaku, Tokyo, Japan), plus 0.02% EDTA in CA^{2+}-, Mg^{2+}- free phosphate buffer saline [PBS(-)]. A total of 5×10^4 cells/dish (Falcon Culture Plastics, Los Angeles, CA) were cultured in 2 ml α-MEM containing 10% FBS. After incubation of cells for 3 days, the medium was removed and fresh medium containing an appropriate concentration of $CdCl_2$ and/or $ZnSO_4$ (Wako Pure Chemicals, Japan) was added. The cells were then cultured for 7 more days with metal ions. During culture, the media were renewed every 3 days.

Culture of bone marrow cells. Eight- to 9-week-old male mice, ddy strain, were obtained from Shizuoka Experimental Animal Center (Shizuoka, Japan). The bone marrow cells were cultured as described by Takahashi et al. (1988) with minor modifications. The femurs and tibiae were dissected out from mice and adhering tissues were cleaned off. The bone ends were cut off and the marrow cells were flushed with α-MEM using a 21-gauge needle. The marrow cells were then cultured in α-MEM containing 10% fetal calf serum (Wako Pure Chemicals, Japan) and 10^{-8} M 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] (Wako Pure Chemicals, Japan) in 24-well plates. A total of 7.5×10^5 cells/500 µl was plated onto each well and cultured at 37° C in a fully humidified atmosphere of 5% CO_2 in air for 8 days. Various concentrations of $CdCl_2$ and/or $ZnSO_4$ were added at the beginning of the culture and at each medium change. During cultures, the media were renewed every 3 days. After culture for 8 days, the cultures were rinsed with PBS(-) and fixed with ethanol-acetone (50 : 50; vol/vol) for 1 min. After the culture plates were dried at room temperature, the cells were stained for tartrate resistant acid phosphatase (TRACP), a marker enzyme for osteoclasts, and counterstained with methyl green. The cells containing three or more nuclei were counted as multinucleated cells (MNC). Multinuclearity and TRACP positivity have been used for identification and analysis of osteoclastic differentiation from bone marrow cells.

Assay of ALP activity and protein content. After appropriate periods of cultivation, cells were washed three times with PBS(-), scraped into 0.2% Nonidet p-40 containing 1 mM $MgCl_2$, and stored at –20° C until measurement of ALP activity and protein content. Cells were sonicated for 4 min with a sonicator (Ultrasonic Generator Model US-50, Nissei). The sonicates were centrifuged for 15 min at 3000 rpm and the supernatants were used for the enzyme assay. ALP activity was assayed by the method of Lowry et al. (1954). The reaction mixture (0.5 ml) consisted of 0.6 M α-amino-2-methylpropanol (pH 10.0), 2.4 mM $MgCl_2$, 9.6 mM *p*-nitrophenyl phosphate as the substrate, and enzyme solution (100 µl). After incubation at 37° C for 30 min, the reaction was stopped by the addition of 1.5 ml 0.25 N NaOH. The amount of *p*-nitrophenol produced was measured in terms of the absorbance at 410 nm. One unit was defined as the enzyme activity causing the release of 1 µmol product per minute. Protein content was measured by using the Pierce BCA protein assay reagent with bovine serum albumin as a standard (Smith et al. 1985). Sample (100 µl) and protein assay dye reagent (2 ml) were mixed. The amount of dye bound to the protein was measured by absorbance at 562 nm versus a reagent blank.

Measurement of hydroxyproline content. Cells were washed three times with PBS(-), scraped off the dishes, and centrifuged at 3000 rpm for 15 min. The pellets were suspended in 1 ml 6 N HCl and hydrolyzed under vacuum in a sealed glass tube at 110° C for 18 h. The hydrolysate was then evaporated, dissolved in 1 ml distilled water, and adjusted to pH 6–8 with 0.01 M KOH. Hydroxyproline content of the sample was determined by the method of Kivirriko et al. (1967).

Mesurement of DNA content. Stored cells were scraped off into 1 ml 1 N $HClO_4$ per dish and kept at 70° C for 20 min. After centrifugation at 3000 rpm for 15 min, pellets were re-extracted with 1 ml 1 N $HClO_4$ at 70° C for 20 min. The combined acid extractable supernatant was used for measurement of DNA content, which was carried out using indole reagent (Ceriotti et al. 1955). To 1 ml acid extractable supernatant was added 0.5 ml 2.5 N HCl and 0.5 ml 0.06% indole, and the mixture was boiled for 10 min. After extraction with 4 ml n-amyl acetate twice, absorbance at 490 nm of the water phase was measured. Calf thymus DNA was used as a standard.

Table 1. Effect of various concentrations of cadmium on ALP activity of MC3T3-E_1

Cd concentration	ALP activity	
	mU/dish	% of control
Control	94.6±6.0	100.0
10^{-8} M	81.2±4.8	85.8
10^{-7} M	69.6±2.5*	73.6
10^{-6} M	66.0±1.9*	69.8
10^{-5} M	3.9±0.4***	4.1

Data represent the mean ± SEM of triplicate cultures
* $p < 0.05$ Significantly different from control culture
*** $p < 0.001$ Significantly different from control culture

Table 2. Effect of various concentrations of cadmium on DNA content of MC3T3-E_1

Cd concentration	DNA content	
	µg/dish	% of control
Control	68.2	100.0
10^{-8} M	75.3	110.5
10^{-7} M	74.9	109.9
10^{-6} M	64.0	93.9
10^{-5} M	42.8	62.8

Each value represents the average of duplicate cultures

Enzyme histochemistry. Staining for TRACP was performed according to the modified method of Burstone (1958) by incubating the fixed cells for 30 min at room temperature in an acetate buffer (0.1 M sodium acetate, pH 5.0) containing naphthol AS-MX phosphate (Sigma Chemical Co., St Louis, MO) as a substrate and red violet LB salt (Sigma Chemical Co., St Louis, MO) as a stain for the reaction product in the presence of 10 mM sodium tartrate (Minkin 1982). TRACP-positive cells appeared as dark red cells.

Statistical analysis. Data were expressed as the mean ± SEM of three or four cultures. The results were analysed for statistical significance by the Student's *t* test.

Results

Table 1 shows the effect of various concentrations of Cd on the activity of ALP, a marker enzyme for osteoblasts, of MC3T3-E_1 cells which were cultured for 7 days after treatment with Cd on day 3 of culture. Although 10^{-8} M Cd did not have a significant effect on ALP activity of the cells, 10^{-7} M and 10^{-6} M Cd significantly decreased ALP activity to 73.6% and 69.8% of the control value, respectively. Addition of 10^{-5} M Cd further decreased the enzyme activity to 4.1% of control value.

Table 2 shows DNA contents of MC3T3-E_1 exposed to various concentrations of Cd which was added on day 3 of the cultivation. Cadmium 10^{-8} M, 10^{-7} M, and 10^{-6} M had no influence on DNA content of MC3T3-E_1. Cd at 10^{-5} M decreased DNA content to 62.8% of the control value.

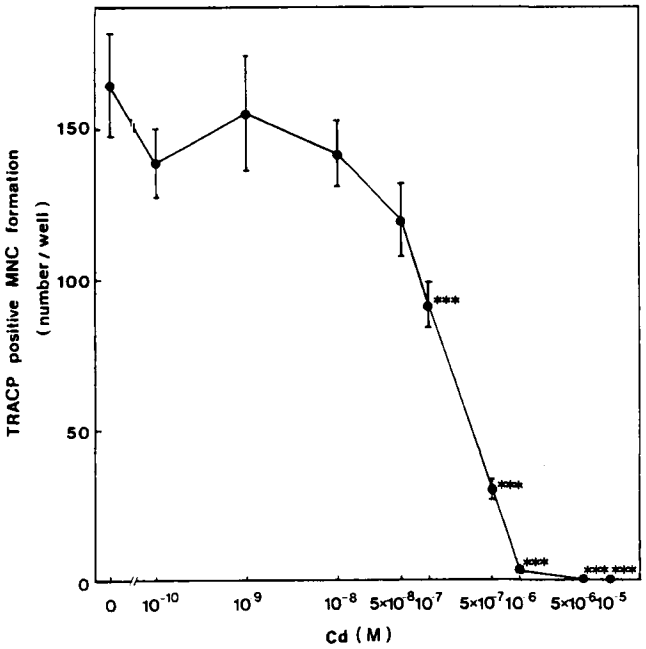

Fig. 1. Effect of varying concentrations of cadmium on the formation of TRACP(tartrate-resistant acid phosphatase)-positive MNC(multinucleated cells) induced by $1\alpha,25(OH)_2D_3$. Data represent the mean ± SEM of four cultures. Significantly different from control (0), *** $p < 0.01$

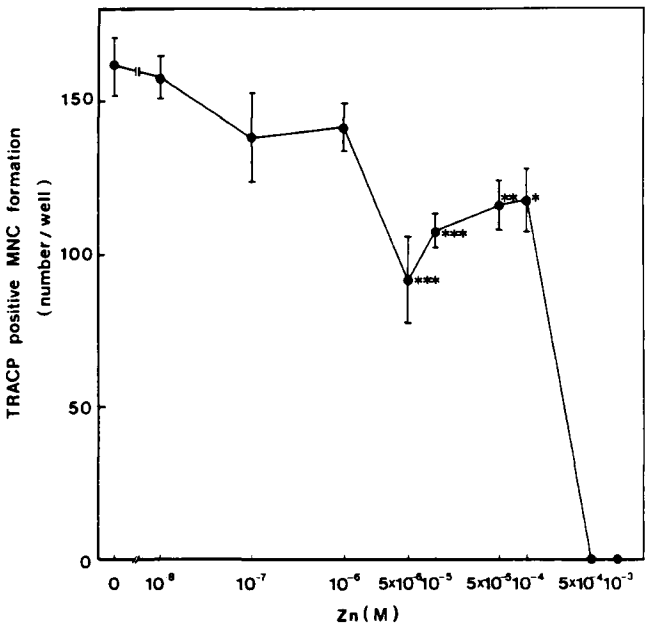

Fig. 2. Effect of varying concentrations of Zn on the formation of TRACP-positive MNC induced by $1\alpha,25(OH)_2D_3$. Data represent the mean ± SEM of four cultures. Significantly different from control (0). * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$

Table 3. Effect of various concentrations of cadmium on hydroxyproline content of MC3T3-E_1

Cd concentration	Hydroxyproline content	
	μg/dish	% of control
Control	66.30	100.0
10^{-8} M	62.34	94.0
10^{-7} M	63.10	95.2
10^{-6} M	61.46	92.7
10^{-5} M	6.23	9.4

Each value represents the average of duplicate cultures

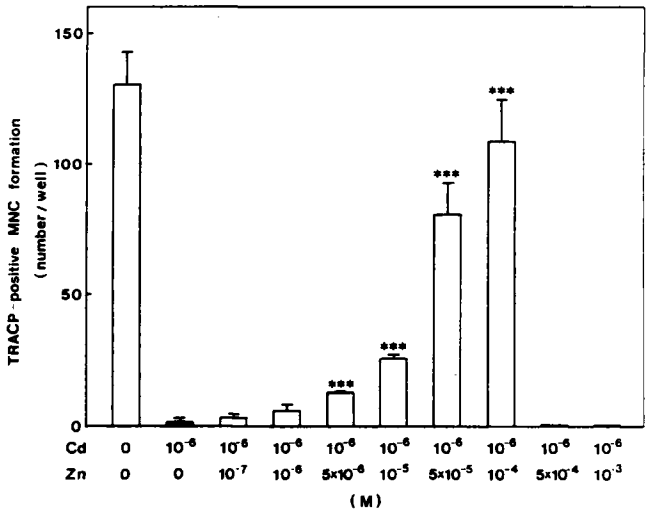

Fig. 3. Effect of varying concentrations of Zn on the TRACP-positive MNC formation inhibited by 10^{-6} M Cd. Data represent the mean ± SEM of four cultures. Significantly different from the culture exposed to 10^{-6} M Cd alone. *** $p < 0.01$

To elucidate the effect of Cd on collagen content, the hydroxyproline content of MC3T3-E_1 exposed to various concentrations of Cd for 7 days was measured. Although 10^{-8} M, 10^{-7} M and 10^{-6} M Cd had no influence on the hydroxyproline content of MC3T3-E_1, 10^{-5} M Cd markedly decreased the hydroxyproline content to 9.4% of the control value (Table 3).

To examine the effect of Cd on bone resorption, the effect of various concentrations of Cd on the formation of TRACP-positive MNC induced by $1\alpha,25(OH)_2D_3$ was investigated (Fig. 1). Mouse marrow mononuclear cells were cultured with increasing concentrations of Cd in the presence of 10^{-8} M $1\alpha,25(OH)_2D_3$. At the higher concentration of 10^{-7} M Cd significantly reduced the number of TRACP-positive MNC. TRACP-positive MNC were not formed by the addition of concentrations of Cd higher than 10^{-6} M Cd.

Since osteoblastic function inhibited by Cd was restored by the simultaneous addition of Zn to the culture medium, as previously reported (Iwami et al. 1992), we examined whether the formation of osteoclasts inhibited by Cd is also prevented by the simultaneous addition of Zn to the culture medium. The effect of Zn alone on MNC formation is shown in Fig. 2. Concentrations of Zn higher than 5×10^{-6} M inhibited TRACP-positive MNC formation significantly and Zn at 5×10^{-4} inhibited it completely. TRACP-positive MNC formation was not stimulated by Zn but was reduced by the addition of a high concentration of Zn to the culture medium.

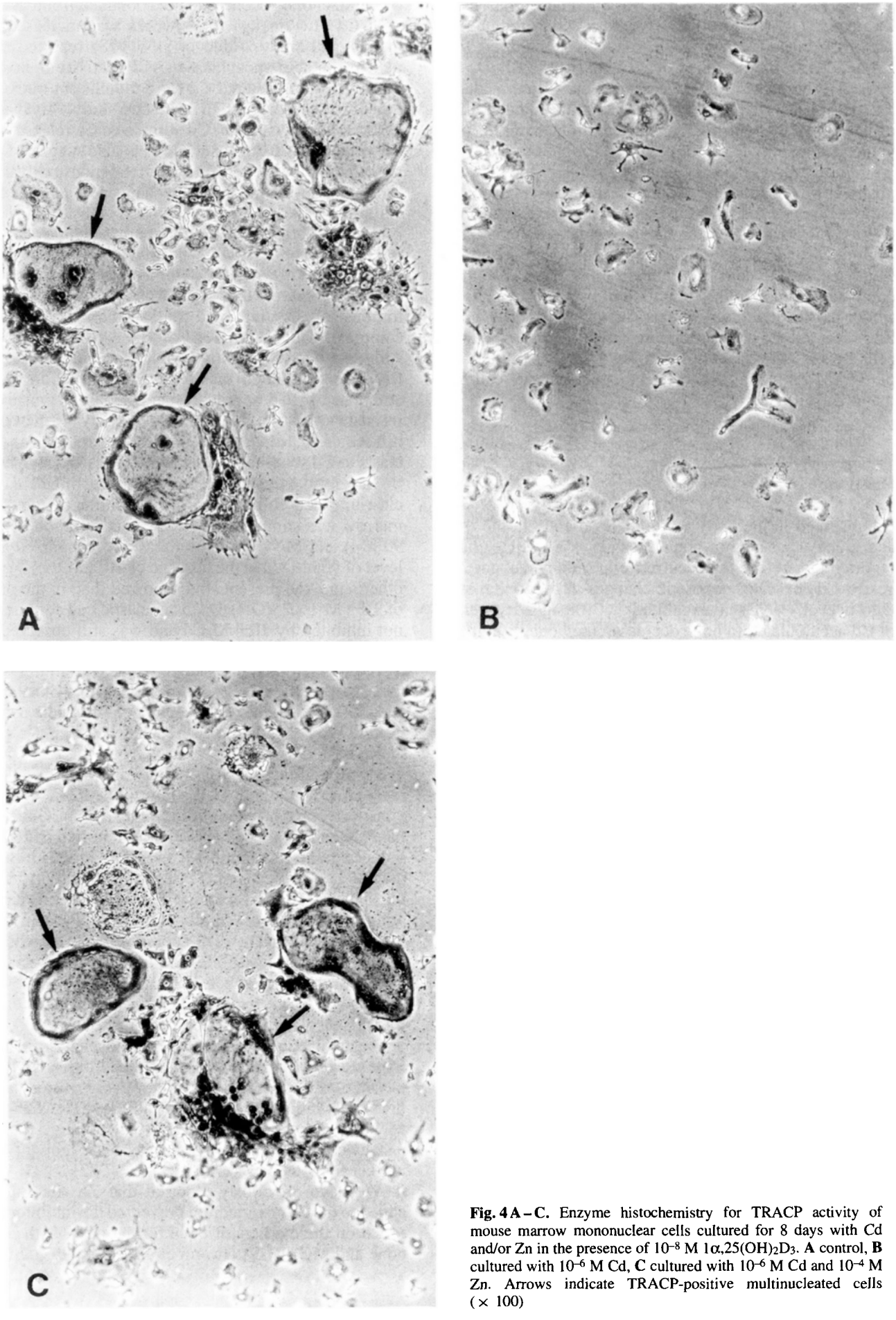

Fig. 4 A – C. Enzyme histochemistry for TRACP activity of mouse marrow mononuclear cells cultured for 8 days with Cd and/or Zn in the presence of 10^{-8} M $1\alpha,25(OH)_2D_3$. **A** control, **B** cultured with 10^{-6} M Cd, **C** cultured with 10^{-6} M Cd and 10^{-4} M Zn. Arrows indicate TRACP-positive multinucleated cells ($\times$ 100)

Figure 3 shows the effect of varying concentrations of Zn on the TRACP-positive MNC formation inhibited by 10^{-6} M Cd. Mouse mononuclear cells were cultured with Cd and Zn in the presence of 10^{-8} M $1\alpha,25(OH)_2D_3$ for 8 days. Although 10^{-6} M Cd completely inhibited the TRACP-positive MNC formation, simultaneous addition of Zn, at a 100-fold higher concentration than Cd, prevented Cd toxicity to osteoclastic cell formation.

Figure 4 shows the enzyme histochemistry for TRACP-positive MNC from mouse bone marrow cultured for 8 days. Figure 4*A* shows the typical enzyme histochemistry of control culture of mouse bone marrow for 8 days. Multinucleated and TRACP-positive cells were identified as osteoclastic cells. Although 10^{-6} M Cd almost completely inhibited the formation of osteoclastic cells by a bone marrow culture system as shown in Fig. 4*B*, 10^{-4} M Zn prevented the decrease in the formation of osteoclastic cells by 10^{-6} M Cd as shown in Fig. 4*C*. A concentration of 10^{-4} M Zn alone had no effect on osteoclastic cell formation histologically.

Discussion

Cd absorbed in the body accumulates mainly in the liver and kidney, but also in bone. Yoshiki et al. (1975) have reported that before the occurrence of kidney damage, ingested Cd causes osteoporotic changes in rat bone tissue. Ogoshi et al. (1992) have reported that 100 ng/g dry weight of Cd accumulated in bone decreased the bone strength of femurs from rats orally administered $CdCl_2$ solution. These data suggest that there is a possibility that Cd accumulated in bone affects the bone metabolism directly.

To investigate the effect of Cd on bone formation, we examined the effect of Cd on the markers of bone formation in established osteoblastic cells, MC3T3-E_1. This cell line isolated from mouse calvaria is a normal cell line which retains osteoblastic functions (Sudo et al. 1983). Following the addition of Cd at day 3 of cultivation to MC3T3-E_1, ALP activity, collagen content and DNA content were decreased after 7 days. After exposure to 10^{-5} M Cd for 10 days altogether, the decrease in ALP activity was much greater than the decrease in collagen and DNA content. Although ALP activity was decreased significantly by the addition of 10^{-7} M Cd (Table 1), collagen content and DNA content were not reduced by exposure to 10^{-7} M Cd (Tables 2, 3). These results coincide with the data from Angle et al. (1990). They also showed that collagen and DNA are more resistant than ALP to Cd toxicity using the 24-h culture of the osteoblastic, osteosarcoma cell line, ROS 17/2.8 in a serum free medium. We have reported that the bone formation in cultured fetal rat calvaria was inhibited by Cd (Iwami et al. 1990). Kaji et al. (1988) also showed that Cd inhibits the bone formation of embryonic chick tibia, and Miyahara et al. (1986, 1988) reported the inhibition of bone calcification of MC3T3-E_1 exposed to Cd for 24 h and 8 days. These data, including our own, suggest that Cd inhibits bone formation directly.

Some reports suggest the stimulation of bone resorption by Cd in vivo and in vitro. Furuta (1978) has reported that the number of osteoclasts in tibia obtained from rat injected with Cd (1.7 mg/kg) for 3 weeks was increased by the histological study. Ando et al. (1978) reported that the increase in acid phosphatase (ACP) activity in bone from rats orally administered Cd for 6 months showed stimulation of bone resorption. In an in vitro study, Miyahara et al. (1980a, b) reported that Cd stimulates Ca release from rat calvarial bone. Suzuki et al. (1989a) also suggested that Cd stimulates the bone resorption of fetal mouse calvaria via a prostaglandin E_2-mediated mechanism. Using the present culture, we clarified the effect of Cd on osteoclasts, which is related to bone resorption more directly, through counting the number of TRACP-positive MNC. In the presence of $1\alpha,25(OH)_2D_3$, 10^{-7} M Cd decreased the formation of TRACP-positive MNC significantly, and higher concentrations of Cd inhibited the formation of osteoclast completely. Concentrations of 10^{-10}–10^{-8} M Cd did not stimulate TRACP-positive MNC formation (Fig. 1). At first, we speculated that osteoclast formation might be stimulated by Cd or at least that osteoclasts must be more resistant than osteoblasts to Cd toxicity. However, such differences between the response of osteoblasts and osteoclasts to Cd were not observed in the present study.

Recently, Miyahara et al. (1991) reported that osteoclast-like cell formation from a culture of mouse bone marrow was stimulated by a low concentration of Cd (60, 90 nM) and MNC formation was not recognized above a level of 90 nM Cd in the absence of $1\alpha,25(OH)_2D_3$. On the other hand, the present study showed that, in the presence of 10^{-8} M $1\alpha,25(OH)_2D_3$, osteoclastic cell formation was not inhibited by 10^{-8} M Cd and was still observed above the level of 10^{-7} M Cd. As Kumegawa et al. (1990) have reported that immature osteoclasts have vitamin D_3 receptors, the possibility of a competitive effect of vitamin D_3 and Cd at the vitamin D_3 receptors on immature osteoclastic cells may explain the discrepancy between our data and those of Miyahara.

Zn alone induced ALP activity and DNA synthesis of rat fetal calvaria and ALP activity of MC3T3-E_1 as previously reported (Iwami et al. 1990, 1992). On the other hand, Yamaguchi et al. (1983) have reported that Zn alone induced bone resorption because Zn administration produced a significant increase in the activity of acid phosphatase in the femoral epiphysis. Therefore, we examined the effect of Zn alone on TRACP-positive MNC formation. However, we did not observe an increase in the number of osteoclasts following the addition of Zn (Fig. 2). These discrepancies between our data and the data of Yamaguchi et al. may result from the differences in the markers by which we judged the bone resorption. Although Yamaguchi et al. measured acid phosphatase activity in bone as a marker of osteoclastic functions, we measured the numbers of TRACP-positive MNC as osteoclasts. By our observation, many cells besides TRACP-positive MNC were stained by the TRACP staining and tartrate resistance of acid phosphatase has been thought to be a marker for osteoclasts (Minkin et al. 1982).

We have previously reported that Zn simultaneously added to the culture medium prevented the inhibitory effect of Cd on the markers of bone formation in both calvarial bone and MC3T3-E_1 (Iwami et al. 1990, 1992). Suzuki et

al. (1989b) have also observed such a phenomenon by the culture of MC3T3-E_1 for 24 h. In the present study, the effect of Zn to the inhibition of TRACP-positive MNC formation by Cd was also examined and was compared with the result obtained from osteoblastic cells. Zn at 10^{-4} M prevented the decrease in TRACP-positive MNC formation by 10^{-6} M Cd. A 100-fold higher concentration of Zn than Cd prevented the toxicity of Cd to osteoclast formation (Figs 3, 4) as observed in our previous experiment on osteoblasts (Iwami et al. 1992). Suzuki et al. (1990) reported that Zn prevented Cd-induced prostaglandin E_2 production and bone resorption by inhibiting Cd accumulation in osteoblasts. Responses of osteoblasts and osteoclasts to Cd in this culture system were the same as mentioned above and the responses of Cd-damaged osteoblasts and osteoclasts to Zn were also the same.

These results suggest that another mechanism by which Cd causes bone damage should be considered, in addition to the induction of osteoclasts. In this study, we used an established cell line derived from mouse calvaria as osteoblasts and primarily cultured cells derived from mouse bone marrow as osteoclasts. To analyze the effects of Cd on bone cells precisely, we are now developing a method to culture both osteoclasts and osteoblasts under the same conditions.

Acknowledgements. This research was supported in part by a Grant-in-aid from the Ministry of Education, Science and Culture, Japan.

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