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# Cadmium Stimulates Osteoclast-like Multinucleated Cell Formation in Mouse Bone Marrow Cell Cultures

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Most of cadmium(Cd)-treated animals have benn reported to show osteoporosis-like changes in bones(Miyahara and Kozuka 1985). This suggests that Cd may promote bone loss by a direct action on bone. Several organ culture systems indicate that Cd stimulates bone resorption(Miyahara et al 1980, Bhattacharyya et al 1988, Suzuki et al 1989a). Suzuki et al(1989b) found that Cd stimulated prostaglandin $E_2$($PGE_2$) production in the osteoblast-like cell, MC3T3-E1. Therefore, they point out that Cd stimulates bone resorption by increasing $PGE_2$ production. It is known that osteoclasts are the primary cells responsible for bone resorption. Osteoblasts also participate in bone resorption. For example, osteoblasts secrete collagenase that degrades uncalcified osteoid and release a factor to activate resting osteoclasts(Akatsu et al 1989a). Recently, several bone marrow cell culture systems have been developed for examining the formation of osteoclast-like multinucleated cells in vitro. $PGE_2$ was shown to stimulate bone resorption by promoting osteoclast formation using a mouse bone marrow cell culture system(Akatsu et al 1989b). As osteoblasts produce $PGE_2$ by Cd-induced cyclooxygenase and may play an important role in osteoclast formation, the present study was undertaken to clarify the possibility that Cd might stimulate osteoclast formation in a mouse bone marrow culture system.

## MATERIALS AND METHODS

Bone marrow cells were cultured by the method of Takahashi et al 1988a). Briefly, the tibia was removed aseptically from 7-wk-old male mice, ddy strain and the epiphysis of the tibia was cut off. The marrow cavity was flushed with 1 mL of $\alpha$-minimum essential medium($\alpha$-MEM: Flow Laboratories, USA). The bone marrow mononuclear cells were then obtained and washed with $\alpha$-MEM and cultured in $\alpha$-MEM containing 10% fetal calf serum(Gibco, USA) at $7.5 \times 10^5$ cells/0.5 mL in 24-well plates(Sumitomo Bakelite Co., Tokyo). The cells were cultured for 7 d at 37°C under 5% $CO_2$ in air and the medium was exchanged at 3 d after inoculation.

Tartrate-resistant acid phosphatase(TRACP) was used as a marker for osteoclasts. Staining for TRACP was performed

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according to the modified method of Burstone(1958). Briefly, cells were washed with a phosphate-buffered saline solution and fixed with ethanol-acetone(50:50 v/v) for 1 min. The fixed cells were incubated for 20 min at room temperature in 0.1 M acetate buffer containing 10 mM sodium tartrate (pH 5.0) and naphthol AS-MX phosphate(Sigma, USA) and fast red violet LB salt(Sigma). TRACP-positive multinucleated cells(MNCs) containing three or more nuclei were considered to be osteoclasts. Results were expressed as the mean±SEM of 8 cultures. Significance of the difference was examined using the Student's t-test.

Osteoclasts have been demonstrated to possess abundant calcitonin(CT) receptors by an autoradiographic technique using [$^{125}$I]-CT. To examine whether bone marrow-derived MNCs induced by Cd possess CT receptors, we also employed the same autoradiographic technique(Takahashi et al 1988b) for examining the cellular binding of [$^{125}$I]-human CT. Briefly, marrow cells were cultured with 90 nM for 7 d on coverslips(Sumitomo) in a 24-well plate. The cells were cultured in the absence of Cd for the last 1 hr. After culture, the cells were incubated with [$^{125}$I]-CT(Amersham, UK) in $\alpha$-MEM containing 0.1% bovine serum albumin for 1 hr at 22°C. Nonspecific binding was assessed in the presence of an excess amount(2 U) of unlabeled CT(Toyo Jozo, Japan). After incubation, the cells were washed with cold $\alpha$-MEM and fixed with 0.1 M cacodylate buffer(pH 7.4) containing 2% formaldehyde and 2% glutaraldehyde. The cells were treated with ethanol-acetone(50:50 v/v) and stained for TRACP. The coverslips were dipped in NR-M2 emulsion (Konishiroku Photo Industry Co., Tokyo), incubated in the dark at for 2wk, and then developed.

## RESULTS AND DISCUSSION

An effect of Cd on MNC formation is shown in Table 1(isobutylmethylxanthine(IBMX)[-]). A significant stimulation of MNC formation was observed at 60 nM Cd or greater. At 90 nM Cd, the stimulation of MNC formation decreased but still remained significant. Above the levels of 90 nM Cd, MNC formation was not recognized. The number of MNC induced by 60 nM Cd was less than that(75.5±6.3) induced by. 33 nM 1,25-dihydroxyvitamin $D_3$(1,25(OH)$_2D_3$) in the experiment performed simultaneously.

Table 1. Effects of Cd on MNC formation in the absence or presence of IBMX

| Cd(nM) | Number of MNC/culture IBMX(2.5 μM) | |
|---|---|---|
| | [-] | [+] |
| 0 | 6.5±1.0 | 20.3±3.3 |
| 30 | 11.3±2.6 | 31.3±6.3$^{a)}$ |
| 60 | 30.1±7.2** | 49.0±4.3### $^{a)}$ |
| 90 | 16.9±2.4*** | 37.7±4.4## $^{b)}$ |

Bone marrow cells were cultured with Cd in the absence or presence of IBMX for 7 d. Each value shows the mean±SEM for 8 cultures. Significantly different from the control (IBMX[-]),**p<0.01,***p<0.001. Significantly different from the control(IBMX[+]),##p<0.01,###p<0.001. Significantly different from IBMX[-],$^{a)}$p<0.05,$^{b)}$p<0.01.

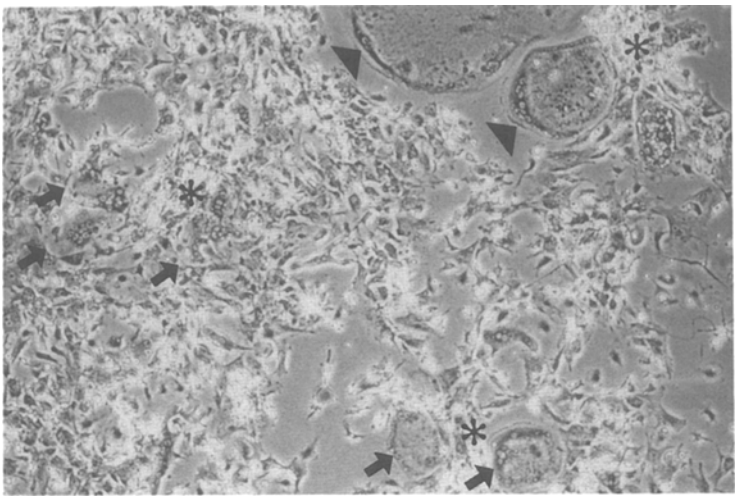

Figure 1. TRACP-positive multinucleated cells formed in mouse bone marrow cultures(x200).
Small multinucleated cells(MNCs, arrows) were observed with large MNCs(triangles). TRACP-positive MNCs were found adjacent to osteoblast-like cells(asteriks).

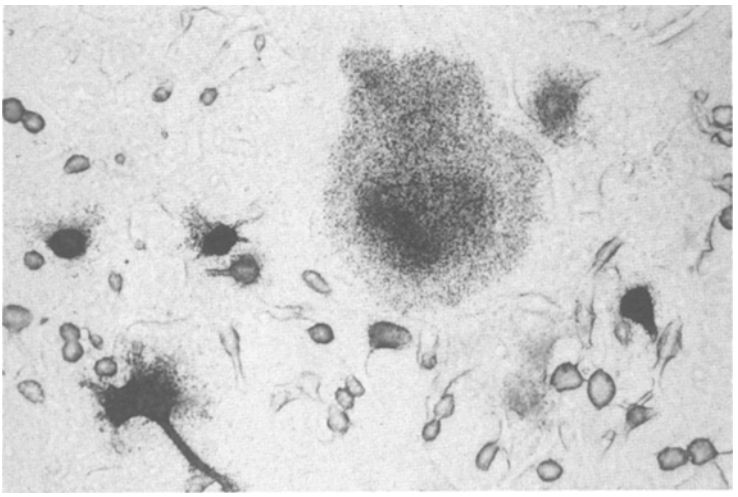

Figure 2. Autoradiogram of [$^{125}$I]-calcitonin binding to mouse bone marrow cell cultures.
Note the numerous dense grains on TRACP-positive MNCs induced by Cd. The grains were completely abolished by adding excess unlabeled calcitonin.

Figure 1 shows TRACP-positive MNCs in bone marrow cells treated with 60 nM Cd for 7d. Cd-treated cultures included many more small MNCs(arrows) than large MNCs(triangles). Large MNCs were observed abundantly in 1,25$(OH)_2D_3$- or $PGE_2$-treated cultures. Most of the MNCs were found near alkaline phosphatase-positive mononuclear cells(asteriks, probably osteoblast-like cells).

Figure 2 shows an autoradiogram of [$^{125}$I]-CT binding to bone marrow cell cultures. Numerous dense grains caused by [$^{125}$I]-CT were recognized on TRACP-positive MNCs induced by 90 nM Cd. Simultaneous addition of excess unlabeled CT (2 U) caused a complete disappearance of the grains. This supports that Cd-induced MNCs are osteoclasts.

To examine Cd-stimulated MNC formation is due to $PGE_2$ production, we used indomethacin that depressed $PGE_2$ production by inhibiting cyclooxygenase. Cd-stimulated MNC formation was inhibited completely by 0.1 μM indomethacin. For example, the number of MNC formed by 75 nM Cd was 19.4±4.0($p<0.01$, vs. control(2.4±1.3), but the number of MNC in the presence of indomethacin was 0. This suggests that Cd-induced osteoclast formation may be due to $PGE_2$.

Osteoclast formation was enhanced over a wide range of exogenous $PGE_2$(10 nM - 10 μM)(Akatsu et al 1989b) but was stimulated within a narrow range of Cd(60-90 nM) (Table 1). As Cd is a strong inhibitor of DNA synthesis, Cd at levels above 90 nM would inhibit proliferation of immature bone marrow cells and thus might not induce osteoclast formation.

As shown in Table 1, 2.5 μM IBMX, an inhibitor of phosphodiesterase, potentiated MNC formation in the absence or presence of Cd. On the other hand, 3 μM verapamil, a calcium channel blocker, depressed Cd-induced MNC formation(Table 2). These results suggest that the activity of Cd in inducing MNC formation may be mediated by the mechanism involving cAMP and $Ca^{2+}$. In addition, as verapamil-binding sites are in osteoblasts(Guggino et al 1988), the inhibitory effect of verapamil on Cd-induced osteoclast formation suggests the dependence of osteoblast-like cells upon osteoclast formation.

Table 2. Effect of verapamil on Cd-stimulated MNC formation

| Cd(nM) | Number of MNC/culture verapamil(3 μM) | |
|---|---|---|
| | [-] | [+] |
| 0 | 14.8±2.7 | 15.1±5.3 |
| 60 | 38.1±5.7** | 20.9±5.0 [b)] |
| 75 | 28.9±4.1** | 14.6±1.8 [b)] |

Bone marrow cells were cultured with Cd in the absence or presence of verapamil for 7 d. Each value represents the mean±SEM for 8 cultures. Significantly different from the control(verapamil [-],**$p<0.01$. Significant difference between verapamil[-] and verapamil[+],[b)] $p<0.01$.

From these results, Cd was shown to promote osteoclast formation. As Cd-stimulated MNC formation was inhibited by indomethacin, endogenous $PGE_2$ was suggested to cause MNC formation. This may be supported by the following reports: 1) Cd stimulates $PGE_2$ production through the induction of cyclooxygenase in osteoblasts(Suzuki et al 1989b). 2) Exogenous $PGE_2$ stimulates osteoclast formation at 10 nM and above(Akatsu et al 1989b).
On the other hand, we recognized that Cd at 0.5 μM or greater stimulated bone resorption using a mouse neonatal parietal bone(in preparation). The concentrations

of Cd causing osteoclast formation were about one-tenth as low as those causing bone resorption. This suggests that Cd-stimulated bone resorption may be due at least partly to the enhancing effect of Cd on osteoclast formation.

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REFERENCES

Akatsu T, Ngata N, Takahashi N, Suda T (1989a) Regulation system of osteoclast. Experimental Med 7: 1272-1278

Akatsu T, Takahashi N, Debari K, Morita I, Murota S, Nagata N, Takatani O, Suda T (1989b) Prostaglandins promote osteoclast-like cell formation by a mechanism involving cyclic adenosine 3',5'-monophosphate in mouse bone marrow cell cultures. J Bone Mineral Res 4:29-35

Bhattacharyya MH, Whelton BD, Stern PH, Peterson DP(1988) Cadmium accelerates bone loss in ovariectomized mice and fetal rat limb bones in culture. Proc Natl Acad Sci USA 85:8761-8765

Burstone MS (1958) Histochemical demonstration of acid phosphatase with naphthol AS-phosphate. J Natl Cancer Inst 21:523-539

Guggino SE, Wagner JA, Snowman AM, Hester LD, Sacktor B, Snyder SH (1988) Phenylalkylamine-sensitive calcium channnels in osteoblast-like osteosarcoma cells. J Biol Chem 263: 10155-10161

Miyahara T, Kozuka H (1985) The direct effect of cadmium on bone metabolism and interaction between cadmium and zinc or copper in tissue culture. Eisei Kagaku 31: 59-71

Miyahara T, Miyakoshi M, Saito Y, Kozuka H (1980) Influence of poisonous metals on bone metabolism III. The efect of cadmium on bone resorption in tissue culture. Toxicol Appl Pharmacol 55: 477-483

Suzuki Y, Morita I, Yamane Y, Murota S(1980a) Cadmium stimulates prostaglandin $E_2$ production and bone resorption in cultured fetal mouse calvaria. Biochem Biophys Res Commun 158: 508-513

Suzuki Y, Morita I, Ishizaki Y, Yamane Y, Murota S (1989b) Cadmium stimulates prostaglandin $E_2$ synthesis in osteoblast-like cells, MC3T3-E1. Biochim Biophys Acta 1012: 135-139

Takahashi N, Yamana H, Yoshiki S, Roodman GD, Mundy GR, Jones SJ, Boyde A, Suda T (1988a) Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. Endocrinology 122: 1373-1382

Takahashi N, Akatsu T, Sasaki T, Nicholson GC, Moseley JM, Martin TJ, Suda T (1988b) Induction of calcitonin receptor by 1$\alpha$,25-dihydroxyvitamin $D_3$ in osteoclast-like multinucleated cells formed from mouse marrow cells. Endocrinology 123: 1504-1510

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