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Reduction of Bone Loss by Denbufylline, an Inhibitor of Phosphodiesterase 4

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ABSTRACT. The effects of denbufylline, a xanthine derivative with selective inhibitory activity on the phosphodiesterase (PDE) 4 isoenzyme, on bone loss in Walker 256/S-bearing rats and on mineralized nodule formation and osteoclastlike cell formation in bone marrow culture systems were examined. Serial oral administrations of denbufylline inhibited the decrease in the bone mineral density of femurs from Walker 256/S-bearing rats, without influence on the healthy rats. Denbufylline restored the bone mass and the number of osteoclasts and osteoblasts per trabecular surface in the femur metaphysis. Among PDE inhibitors, only PDE4-selective inhibitors increased the number of mineralized nodules and decreased the number of osteoclastlike cells in the *in vitro* bone marrow culture systems, and dibutyryl cyclic AMP mimicked these effects in the *in vitro* systems. These results suggest that the PDE4 isoenzyme may play an important role in bone turnover through cyclic AMP and that its inhibitors are candidates for therapeutic drugs for the bone loss diseases. BIOCHEM PHARMACOL **54**;5:613–617, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. PDE4; denbufylline; Walker 256; osteoporosis; bone marrow culture

Osteoporosis represents a major health problem, especially for postmenopausal and aged women. Although there do exist some drugs for treatment of osteoporosis, these drugs seem to be insufficient to improve the quality of life of osteoporotic patients [1]. Additionally, more available and simpler osteoporosis model systems are also being sought for evaluation of drug effects. We recently reported that Walker 256/S carcinosarcoma, which is a variant lacking bone metastasis, caused osteoporosislike changes in rats within 2 weeks, accompanied by decreased serum β-estradiol levels [2]. We found that a xanthine structure phosphodiesterase (PDE#) 4 inhibitor, denbufylline [3], inhibited osteoporosislike changes in Walker 256-bearing rats via oral administration. This paper describes the effects of denbufylline on osteoblast and osteoclast formation *in vitro* and *in vivo*.

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Abbreviations: BMD, bone mineral density; IBMX, 3-isobutyl-1-methylxanthine; MNC, multi-nucleated cell; $1\alpha,25(OH)_2D_3$, $1\alpha,25$-dihydroxyvitamin D_3; PDE, phosphodiesterase; PTHrP, parathyroid hormone-related peptide; TRAP, tartrate-resistant acid phosphatase.

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MATERIALS AND METHODS

Experiments on Walker 256/S-Bearing Rats

Walker 256/S carcinosarcoma was maintained by serial (2-week interval) subcutaneous transplantations in female Wistar Imamichi rats (6 weeks old, Imamichi Institute for Animal Reproduction, Ibaraki, Japan). Animals were housed under special pathogen-free conditions at 24–25°C and 55% humidity with a circadian light rhythm of 12 hr, standard diet pellets (Oriental Yeast Co., Tokyo, Japan), and tap water *ad lib*. Five animals in a group were subcutaneously inoculated in the back with 1 mm^3 of the carcinoma from donors. From 1 day after transplantation, denbufylline dissolved in water was orally administered once a day for 13 days. Animals were killed by decapitation 14 days after the start of experiments, and femurs of hind limbs were removed and fixed in 10% phosphate-buffered formalin. The bone mineral density (BMD) was measured using a dual-energy X-ray absorptiometer (DCS-600R, Aloka, Tokyo, Japan). Thereafter, the femur bone specimens were prepared into undecalcified and decalcified sections, according to common procedures. The preparations were stained for tartrate-resistant acid phosphatase (TRAP) and counterstained with toluidine blue. Then, morphometric parameters of bone were measured by using a texture analyzing system (TAS plus, Ernst Leitz, Germany).

Bone Marrow Culture

Rat and mouse bone marrow cells were prepared for *in vitro* mineralization [4–6] and osteoclastlike cell formation [7, 8], respectively, according to reported methods. Cells were basically cultured in α-MEM (Gibco Lab., Grand island, NY) supplemented with 10% fetal bovine serum (Moregate, Victoria, Australia) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

For the mineralization culture of rat bone marrow, bone marrow cells from femurs of 5-week-old male Wistar rats were seeded into 12-well plates (Sumitomo, Tokyo, Japan) at a density of 10^6 cells/cm^2. Twenty-four hours after seeding, the medium was changed to fresh medium supplemented with 0.2 mM ascorbic acid phosphate ester (Wako Pure Chemical, Osaka, Japan), 1 mM β-glycerophosphate (Sigma Chemical Co., St. Louis, MO), and 10 nM dexamethasone (Sigma). The treatment with agents was carried out for 24 hr on day 3, then the medium was changed every 2 days. At day 14, the culture was fixed with methanol and stained with Alizarin red S, and the area and number of mineralized nodules were measured using a computer image analyzer (IBAS, Kontron Electronik, Germany).

For the osteoclastlike cell formation assay, bone marrow cells from femurs and tibias of 8-week-old ddY male mice were seeded into 24-well plates at a density of 1.5×10^6 cells/mL, and medium was changed to fresh medium supplemented with 10 nM 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$, Teijin, Tokyo, Japan) every 2 days. Varying concentrations of agents were added to the culture 24 hr after seeding. At day 8, the cells were fixed and stained for TRAP in 50 mM sodium tartrate, pH 5.0. TRAP-positive cells containing three or more nuclei (i.e. multinucleated cells; MNCs) were counted under light microscopy.

Agents

Denbufylline [1,3-di-*n*-butyl-7-(2′-oxopropyl)xanthine] was synthesized in our laboratory [9]. Dibutyryl cyclic AMP, 3-isobutyl-1-methylxanthine (IBMX), milrinone, and zaprinast were purchased from the Sigma Chemical Co. Ro 20-1724 was obtained from Research Biochemicals Inc., Natick, MA. These agents were dissolved in dimethylsulfoxide and diluted over 1000-fold by culture medium. Dimethylsulfoxide below 0.5% was confirmed not to have any effect in the *in vitro* assay systems.

Statistics

Data are represented as the mean ± SD. Statistical analyses were done by using Student's *t*-test or Welch's *t*-test.

RESULTS

Walker 256/S carcinoma grew to ca. 25 g wet weight in the backs of rats up to 14 days after inoculation and significantly decreased the BMD of femurs of the host animals without bone metastasis or parathyroid hormone-related peptide (PTHrP) production, as previously reported [2]. As shown in Fig. 1, oral administration of denbufylline did not affect the femur growth of healthy rats, but did inhibit the decrease in the BMD in a dose-dependent manner up to 1.0 mg/kg, without influence on the body weight, tumor growth, or behavior of the animals. In histomorphological studies, the primary and secondary trabecular bone mass was clearly decreased in the femurs of Walker 256/S-bearing rats, and denbufylline restored the bone loss. Table 1 shows the data from the histomorphometric analysis of distal metaphysis of the femurs. All parameters from the tumor-bearing rats were improved by the treatment with denbufylline: it was of particular interest that the osteoclast number was increased and the osteoblast number was decreased in the tumor-bearing group, and that these cell numbers were significantly improved by denbufylline (1.0 mg/kg) treatment.

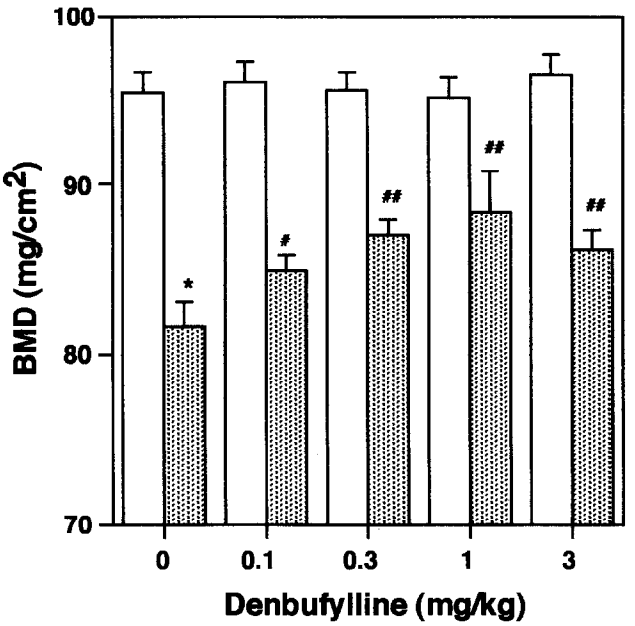

FIG. 1. Effects of denbufylline on the femur BMD of healthy rats and Walker 256/S-bearing rats. The indicated doses of denbufylline were orally administered to age-matched healthy rats (open column) and Walker 256/S-bearing rats (dotted column) once a day for 13 days, and total BMD of femurs was measured at day 14 after the tumor inoculation. Data are represented as the mean ± SD ($n = 5$). *Significantly different from the age-matched healthy control at $P < 0.01$. # and ##Significantly different from the Walker 256/S control at $P < 0.05$ and 0.01, respectively.

Then, to see whether these effects of denbufylline are a specific action on bone cell recruitment due to its PDE4 inhibitory activity, the effects of some PDE isoenzyme inhibitors were examined in the *in vitro* bone marrow culture systems. As shown in Fig. 2, a nonselective PDE inhibitor IBMX and two PDE4 inhibitors, denbufylline and Ro 20-1724, showed a drastic increase in the number of mineralized nodules and a decrease in the number of TRAP-positive MNCs. Other inhibitors of PDE3 (milri-

TABLE 1. Histomorphometric analysis of femur metaphysis

Item		Walker 256/S-bearing rats		
		Healthy control	Vehicle	Denbufylline 1.0 mg/kg, p.o.
Bone volume/tissue volume	%	14.6 ± 2.3	7.90 ± 4.87*	17.7 ± 2.8†
Trabecular number	no./mm	6.52 ± 0.73	5.18 ± 2.35	7.84 ± 0.93
Resorption surface/bone surface	%	28.8 ± 2.0	45.8 ± 9.4*	24.2 ± 5.0††
Osteoclast number/bone surface	cells/mm	10.9 ± 2.1	19.1 ± 1.5**	11.3 ± 2.0†††
Osteoblast number/bone surface	cells/mm	229 ± 25	42.8 ± 16.1**	109 ± 39†

Measurements were done in a ca. 1.7 mm^2 area in metaphysis of femurs.
Data are the mean ± SD (n = 5).
Significantly different from the healthy control at *$P < 0.05$ and **0.001, respectively.
Significantly different from the vehicle control at †$P < 0.05$, ††0.01, and †††0.001, respectively.

none) and PDE5 (zaprinast) at 10 μM were without effect on the bone marrow culture systems. Figure 3 indicates the concentration-dependent actions of denbufylline and no effect of milrinone in both experimental systems. The effects of denbufylline (0.1 μM) were never increased by milrinone (Fig. 3) and zaprinast (data not shown). On the other hand, dibutyryl cyclic AMP, a membrane permeable cyclic AMP analog, mimicked the actions of denbufylline in the *in vitro* culture system (Fig. 4).

DISCUSSION

In this study, we have demonstrated that the oral administration of a PDE4 inhibitor, denbufylline, improved the decrease in the BMD and changes in histomorphometric parameters, including a decrease in the number of osteoblasts and an increase in the number of osteoclasts, in femur bones from rats bearing Walker 256/S carcinosarcoma. Although detailed mechanisms of the osteoporosislike changes induced by Walker 256/S tumor are unclear now, this tumor never secretes PTHrP but causes a significant decrease in the serum level of β-estradiol and an increase in the activity of the reticuloendothelial immunosystem [2]. Therefore, Walker 256/S-bearing rats seem to be a model of osteoporosis caused by hypoovarianism or postmenopause.

This is the first report that the PDE4 inhibitor is effective on osteoporotic disorders without affecting healthy rats. Because inhibitors of PDE4, but not other PDE isoenzymes,

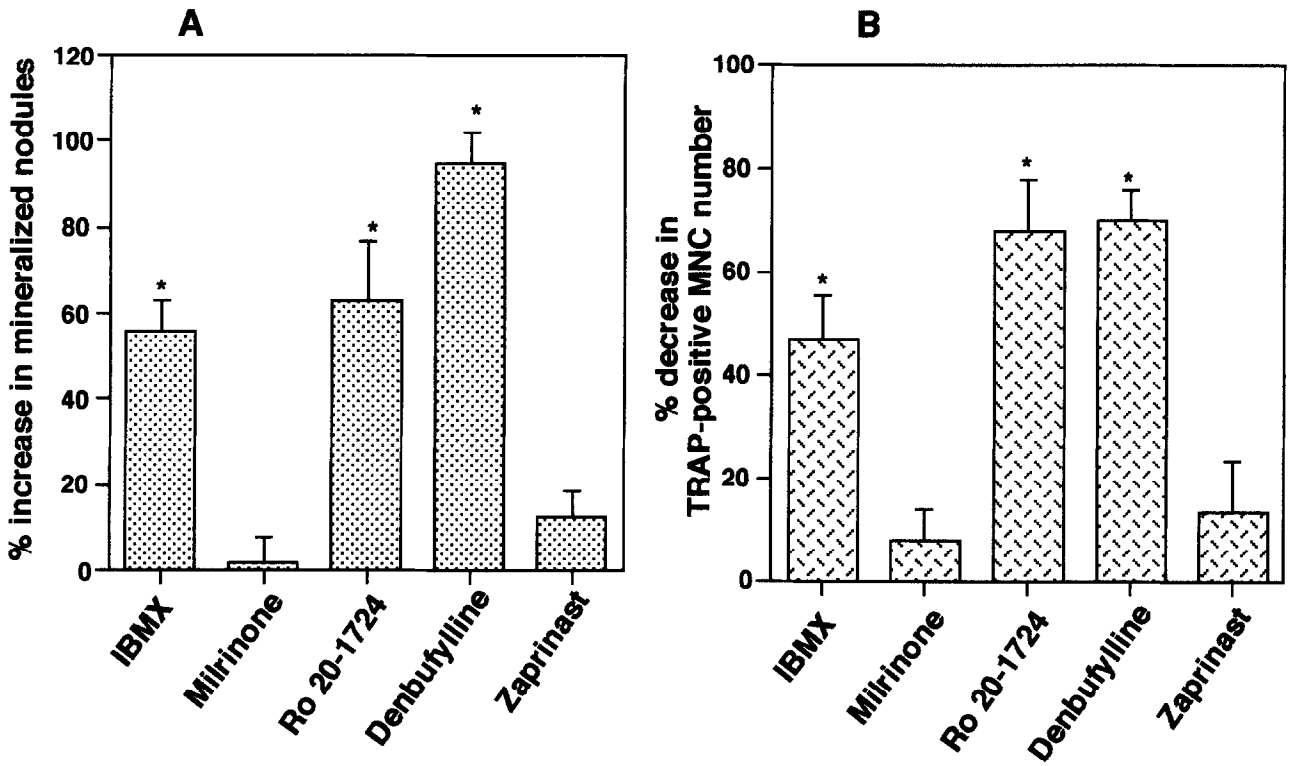

FIG. 2. Effects of PDE inhibitors (each 10 μM) on mineralized nodule formation (A) and TRAP-positive MNC formation (B) in the *in vitro* bone marrow culture. The number of the mineralized nodules and the number of TRAP-positive MNCs in the control cultures were 48.6 ± 8.3 (n = 12) and 121 ± 17 (n = 10), respectively. Data are represented as the mean ± SD (n = 4). *Significantly different from the control culture at $P < 0.001$.

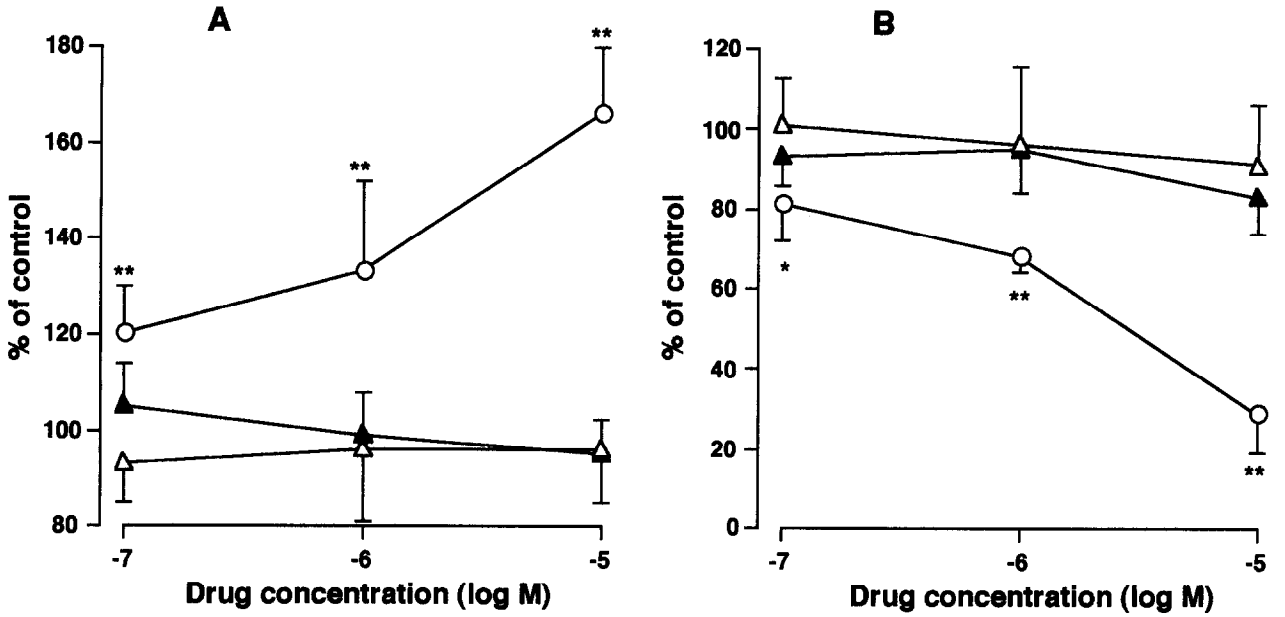

FIG. 3. Effects of denbufylline (○) and milrinone (△) and in combination with 0.1 μM denbufylline and milrinone (▲) on mineralized nodule formation (A) and TRAP-positive MNC formation (B) in the *in vitro* bone marrow culture. Data are represented as the mean ± SD (n = 4–6). * and **Significantly different from the control culture at $P < 0.01$ and 0.001, respectively.

inhibit secretion and gene expression of inflammatory cytokines such as interleukin-1α, interleukin-6, and tumor necrosis factor-α in macrophages [10, 11], the systemic actions of denbufylline may be considered. It is likely that these cytokines are secreted from the bone cells in some cyclic AMP-dependent manner and affect the differentiation of osteoblasts and osteoclasts and their functions. It has been well documented that the generation of osteoblasts and osteoclasts and their activities were regulated by the bone metabolism-related hormones, such as PTH, calcitonin, prostagladins, etc., through the action of cyclic AMP [12–16]. The PDE inhibitors are expected to modulate the actions of these hormones and cytokines in the bone.

We have reported that prostaglandin E_2 or PTH stimulates mineralized nodule formation when these agents are added to rat bone marrow culture in the early culture period, i.e. day 3 or 4 of culture [17, 18]. In this study, among PDE inhibitors only PDE4 inhibitors showed a similar anabolic effect, suggesting a stimulative effect of PDE4 inhibitors on osteoblast differentiation. Moreover,

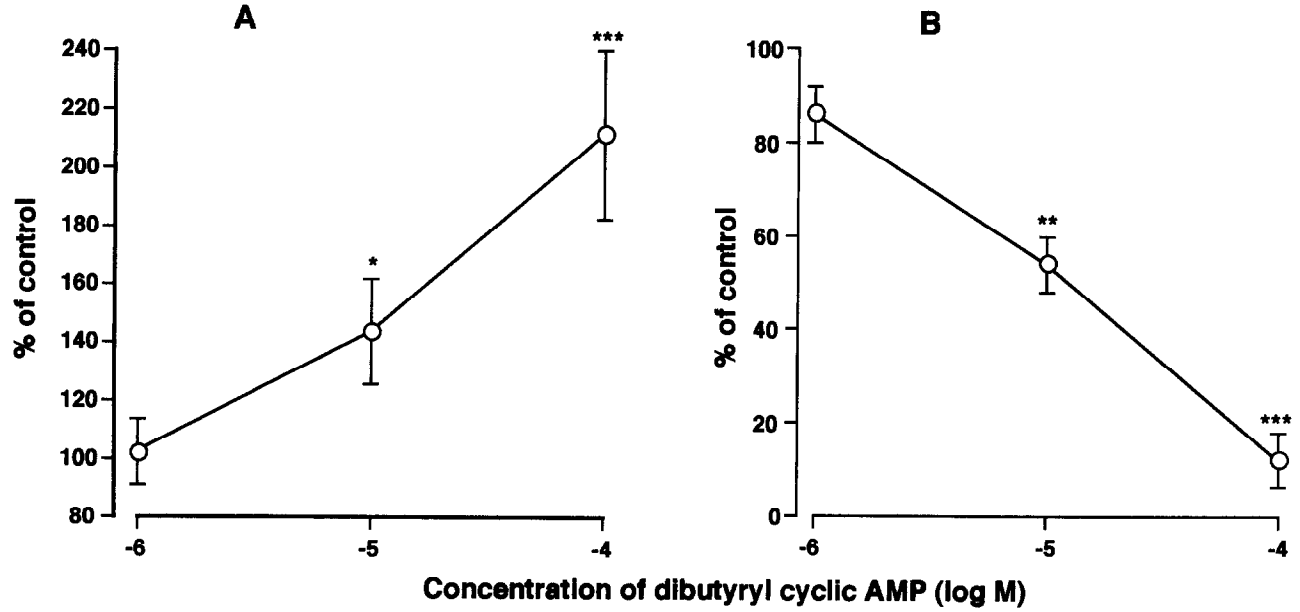

FIG. 4. Effects of dibutyryl cyclic AMP on mineralized nodule formation (A) and TRAP-positive MNC formation (B) in the *in vitro* bone marrow culture. Data are represented as the mean ± SD (n = 4). *, **, and ***Significantly different from the control culture at $P < 0.05$, 0.01, and 0.001, respectively.

PDE4 inhibitors inhibited osteoclastlike cell formation in the mouse bone marrow culture system. The effects of denbufylline were mimicked by dibutyryl cyclic AMP, but were not further increased by combination with other PDE inhibitors. Interestingly, an increase in osteoblast number and a decrease in osteoclast number were observed in Walker 256/S-bearing rats treated with denbufylline. Therefore, the effects of the PDE4 inhibitor seem to be specific for bone diseases, although further experiments are needed to corroborate this.

Consequently, our *in vivo* and *in vitro* results indicate that PDE4 inhibitors could stimulate osteoblast differentiation and inhibit osteoclast formation. This study suggests that the PDE4 isoenzyme is important in cyclic AMP degradation in bone marrow cells and that the PDE4 inhibitors are good candidates as therapeutic drugs for osteoporosis and other bone diseases.

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References

1. Rungby J, Hermann AP and Mosekilde L, Epidemiology of osteoporosis: Implication for drug therapy. *Drugs Aging* **6:** 470–478, 1995.
2. Waki Y, Miyamoto K, Kasugai S and Ohya K, Osteoporosis-like changes in Walker carcinoma 256-bearing rats, not accompanied with hypercalcemia or parathyroid hormone-related protein production. *Jpn J Cancer Res* **86:** 470–476, 1995.
3. Nicholson CD, Jackman SA and Wilke R, The ability of denbufylline to inhibit cyclic nucleotide phosphodiesterase and its affinity for adenosine receptors and the adenosine re-uptake site. *Br J Pharmacol* **97:** 889–897, 1989.
4. Maniatopoulos C, Sodek J and Melcher AH, Bone formation *in vitro* by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res* **254:** 317–330, 1988.
5. Kasugai S, Bone marrow stromal cell culture as a bone formative experimental system: Technical points and applications to bone research. In: *Pharmacological Approach to the Study of the Formation and the Resorption Mechanism of Hard Tissues* (Ed. Ogura H.), pp. 175–186. Ishiyaku EuroAmerica, Tokyo, 1994.
6. Ozawa S and Kasugai S, Evaluation of implant materials (hydroxyapatite, glass-ceramics, titanium) in rat bone marrow stromal cell culture. *Biomaterials* **17:** 23–29, 1996.
7. Takahashi N, Yamada 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.
8. Soekanto A, Ohya K and Ogura H, The effect of sodium salicylate on the osteoclast-like cell formation and bone resorption in a mouse bone marrow culture. *Calcif Tissue Int* **54:** 290–295, 1994.
9. Miyamoto K, Yamamoto Y, Kurita M, Sakai R, Konno K, Sanae F, Ohshima T, Takagi K, Hasegawa T, Iwasaki N, Kakiuchi M and Kato H, Bronchodilator activity of xanthine derivatives substituted with functional groups in the 1- and 7-position. *J Med Chem* **36:** 1380–1386, 1993.
10. Semmler J, Wachtel H and Endres S, The specific type IV phosphodiesterase inhibitors rolipram suppress TNFa production by human mononuclear cells. *Int J Immunopharmacol* **15:** 409–413, 1993.
11. Kambayashi T, Jacob CO, Zhou D, Mazurek N, Fong M and Strassmann G, Cyclic nucleotide phosphodiesterase type IV participates in the regulation of IL-10 and in the subsequent inhibition of TNF-a and IL-6 release by endotoxin-stimulated macrophages. *J Immunol* **155:** 4909–4916, 1995.
12. Partridge NC, Alcorn D, Michelangeli VP, Kemp BE, Ryan GB and Martin TJ, Functional properties of hormonally responsive cultured normal and malignant rat osteoblastic cells. *Endocrinology* **108:** 213–219, 1981.
13. Nijweide PJ, Cells of bone: Proliferation, differentiation, and hormonal regulation. *Physiol Rev* **66:** 855–886, 1986.
14. Nicholson GC, Livesey SA, Moseley JM and Martin TJ, Actions of calcitonin, parathyroid hormones, and prostaglandin E2 on cyclic AMP formation in chicken and rat osteoclasts. *J Cell Biochem* **31:** 229–241, 1986.
15. Yamaguchi DT, Green J, Merritt BS, Kleeman CR and Muallen S, Modulation of osteoblast function by prostaglandins. *Am J Physiol* **257:** F755–761, 1989.
16. Kasugai S, Oida S, Iimura T, Arai N, Takeda K, Ohya K and Sasaki S, Expression of prostagalndin E receptor subtypes in bone: Expression of EP_2 in bone development. *Bone* **17:** 1–4, 1995.
17. Kasugai S, Ozawa S, Kondo H, Mukohyama H, Oida S, Iimura T, Arai N and Ohya K, Prostaglandin E2 effects on mineralized tissue formation *in vitro* depend on the cell stage of osteoblastic lineage. *J Bone Mineral Res* **10:** S420, 1995.
18. Kondo S, Kasugai S, Ozawa S, Mukohyama H, Ohya K and Ohyama T, The effects of parathyroid hormone (PTH) on mineralized tissue formation in rat bone marrow cell culture. *J Bone Mineral Res* **10:** S420, 1995.