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Inhibition of osteoclastogenesis by a phosphodiesterase 4 inhibitor XT-611 through synergistic action with endogenous prostaglandin E_2

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Abstract

We examined the effect of a phosphodiesterase 4 (PDE4) inhibitor, 3,4-dipropyl-4,5,7,8-tetrahydro-3*H*-imidazo[1,2-*i*]-purin-5-one (XT-611) on osteoclast formation in three different mouse bone-marrow cell (BMC) culture systems. We confirmed that selective inhibitors of PDE4, including XT-611, among several PDE inhibitors decreased osteoclast formation in the BMC culture system. XT-611 also inhibited osteoclast formation in co-culture of mouse bone-marrow stromal cell line ST2 and adherent cell-depleted (ACD)-BMCs. However, it did not inhibit osteoclastogenesis in culture of ACD-BMCs alone in the presence of macrophage-colony stimulating factor (M-CSF) and soluble receptor activator of NF-κB ligand (sRANKL). XT-611 significantly increased prostaglandin E_2 (PGE_2) production from ST2 cells and, in combination with PGE_2, synergistically increased cAMP concentration in osteoclast progenitors. In the ST2 co-culture system, XT-611 did not influence the expression of RANKL, osteoprotegerin and RANK mRNAs. By combined treatment with XT-611 and PGE_2 of ACD-BMCs, osteoclast multinucleation was clearly inhibited with decrease in the expression of calcitonin receptor mRNA, while the expression of RANK and c-fms (an M-CSF receptor) mRNAs was unchanged. These results indicate that the PDE4 inhibitor inhibits osteoclastogenesis by acting on osteoclast progenitors synergistically with PGE_2 secreted from stromal cells, but not by influencing the cell-to-cell interaction between stromal cells and osteoclast progenitors.

Keywords: Phosphodiesterase 4 (PDE4) inhibitor; XT-611; Osteoclastogenesis; Prostaglandin E_2 (PGE_2); cAMP

1. Introduction

The signal pathways mediated by cAMP as an intracellular second messenger after stimulation of various receptors are well known to be involved in osteoclastogenesis and osteoblastogenesis. The inhibition of hydrolysis of cAMP produced in response to various hormones is therefore expected to potentiate the hormone actions on bone cells. Cyclic nucleotide PDE is now classified into at least 10 isoenzymes, which differ in their substrate specificity, affinity for cyclic nucleotide, and regulatory properties [1–4]. Among them, PDE4 isoenzyme is a cAMP-specific PDE, and its inhibitors have selective bronchodilator activity [5,6] and anti-inflammatory action [7]. Moreover, we recently reported that PDE4 inhibitors specifically produced an increase of osteoblast formation and a decrease of osteoclast formation in an *in vitro* BMC culture, and we observed a therapeutic effect of PDE4 inhibitors against bone loss in some animal osteopenia models [8,9]. However, the mechanisms of action of PDE4 inhibitors on osteoblastogenesis and osteoclastogenesis are poorly understood.

It is well known that monocytes and macrophage-like stem cells differentiate into osteoclast cells with the support of osteoblast/stromal cells. It has been clarified that

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Abbreviations: PDE, phosphodiesterase; BMC, bone-marrow cell; RANK, receptor activator of nuclear factor κB; RANKL, RANK ligand; OPG, osteoprotegerin; 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; PGE_2, prostaglandin E_2; XT-611, 3,4-dipropyl-4,5,7,8-tetrahydro-3*H*-imidazo[1,2-*i*]-purin-5-one; IBMX, 3-isobutyl-1-methylxanthine; ACD, adherent cell-depleted; M-CSF, macrophage-stimulating factor; MNC, multinucleated cell; TRAP, tartrate-resistant acid phosphatase.

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osteoclast progenitor cells are stimulated and their maturation promoted through a membrane interaction with osteoblasts/stromal cells via the RANK–RANKL (also called osteoclast differentation factor (ODF)) system [10,11]. Osteoclast formation and maturation are promoted by a change in the production ratio of RANKL vs. OPG (a decoy receptor for RANKL secreted from stromal cells; also called osteoclastogenesis inhibitory factor (OCIF)) after stimulation of osteoblastic stromal cells by osteotropic factors such as 1α,25-$(OH)_2D_3$, parathyroid hormone, or IL-11 [12,13]. On the other hand, calcitonin and PGE_2 directly inhibit osteoclast formation and bone resorption activity [14,15]. Therefore, osteoclastogenesis is regulated by interaction with stromal cells and stimulation by several osteotropic factors. As mentioned above, PDE4 inhibitors exhibit complex actions in a BMC culture system. Thus, to clarify the action mechanism of PDE4 inhibitors, the experimental systems should be simplified. In this study, we examined the effects of XT-611, a novel and potent PDE4 inhibitor developed by us [16], on osteoclastogenesis in three experimental systems; mouse BMC culture, co-culture of osteoblastic stromal cell ST2 cells and ACD-BMCs, and single culture of ACD-BMCs.

2. Materials and methods

2.1. Drugs and animals

PDE4 inhibitors XT-611 and rolipram (4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone) were synthesized in our laboratory. IBMX, milrinone, zaprinast, dexamethasone, naphthol AS-MX phosphate sodium, fast red violet LB salt (Sigma Chemical Co.), 1α,25-$(OH)_2D_3$ (Teijin), PGE_2 (Cayman), and bisbenzimide H 33258 (Wako) were purchased from the indicated commercial sources. Recombinant human M-CSF and recombinant human sRANKL were from PeproTech EC. Oligonucleotide primers were custom-synthesized by Amersham Pharmacia Biotech. Male ddY mice (8 weeks old, Nippon SLC) were used randomly in this study. A mouse bone-marrow stromal cell line ST2 was obtained from Riken Cell Bank.

XT-611 inhibits PDE4 activity by 1.6 μM of IC_{50}, but does not other PDE isoenzymes by over 100 μM.

2.2. BMC culture

Femurs and tibias were cut out from mice, and then connective and soft tissues were aseptically removed. The ends of the bones were cut off, and the bone marrow was flushed out into a 50 mL tube with pH 7.4 alpha modification of Eagle's medium (Dainippon) containing 10% heat-inactivated fetal bovine serum (Moregate) and 60 mg/mL kanamycin (Meiji Seika), using a 1 mL syringe. After centrifugation of the cell suspension at 1000 rpm for 5 min, the supernatant was removed, and the cells were resuspended in the medium to make 3×10^6 cells/mL. One milliliter of the suspension was seeded into 24-well plates, and the cells were cultured in a medium supplemented with 10 nM dexamethasone, and 10 nM 1α,25-$(OH)_2D_3$ at 37° for 7 days. The medium was replaced every 3 days with fresh medium. The culture was fixed with 4% *para*-formaldehyde in phosphate-buffered saline solution (pH 7.4) at 4°, and stained in 0.1 mg/mL naphthol AS-MX phosphate sodium, 0.6 mg/mL fast red violet LB salt, 50 mM sodium tartrate/0.1 M sodium acetate, pH 5.0 for TRAP. Nuclei were stained with bisbenzimide H 33258. The sample was washed with distilled water and dried, then the number of TRAP-positive MNCs, containing over five nuclei, was counted under light microscope.

2.3. Preparation of ACD-BMCs

A 2 mL aliquot of BMC suspension was gently passed into a Sephadex G-10 (Amersham Pharmacia Biotech) column system, according to the method of Ly and Mishell [17]. The BMCs were with infiltrated the carrier, then 8 mL of the medium was added, and the cells were incubated at 37° for 45 min. The eluted suspension of ACD-BMCs was collected.

2.4. Co-culture of ST2 cells and ACD-BMCs

ST2 cells were suspended at 2×10^4 cells/mL in alpha modification of Eagle's medium containing 10% fetal bovine serum, 10 nM 1α,25-$(OH)_2D_3$ and 10 nM dexamethasone, and then 250 μL/well of the cell suspension was seeded into 48-well plates. After culture for 72 hr, 250 μL/well of the ACD-BMCs (1.5×10^6 cells/mL) was added in piles. Thereafter, treatment was carried out for 15 days, with replacement of the medium every 3 days, and then the cultures were fixed and stained for TRAP.

2.5. Primary culture of ACD-BMCs

According to the method of Niida *et al.* [18], ACD-BMCs were cultured to generate osteoclasts. Wells of 96-well plates were coated with 5 μL of a solution containing 625 ng/mL of M-CSF and 2.5 μg/mL of sRANKL in fetal bovine serum and dried for 1 hr in a clean bench. The ACD-BMCs were suspended at 1.5×10^6 cells/mL in alpha modification of Eagle's medium containing 25 ng/mL of M-CSF and 100 ng/mL of sRANKL, and then 125 μL/well of the cell suspension was seeded into 96-well plates. At 48 hr into the culture, the medium was changed to alpha modification of Eagle's medium containing 15% fetal bovine serum, 25 ng/mL of M-CSF and 100 ng/mL of sRANKL. Thereafter, treatment was carried out for 5 days, with replacement of the medium every other day, and the cultures were fixed and stained for TRAP.

We confirmed the absence of stromal cells or osteoblast progenitors in the cell population on the basis

that alkaline phosphatase mRNA expression and alkaline phosphatase-positive cells or nodules were never observed during treatment with or without XT-611 under these conditions, though PDE4 inhibitors, including XT-611, exhibit osteoblastogenic activity on stromal cells or BMCs [9].

2.6. Measurement of cAMP and PGE_2

PGE_2 concentration in the culture medium was assayed using a PGE_2 enzyme immunoassay kit (Cayman Chemical). Intracellular cAMP was measured with a cAMP EIA system (Amersham).

2.7. Reverse transcription–polymerase chain reaction assay

mRNAs were prepared from cultures by using a QuickPrep micro mRNA purification kit (Pharmacia Biotech AB). Synthesis of cDNA from the isolated mRNA was carried out using RNase H-reverse transcriptase (Gibco-BRL). Reverse transcription reactions were carried out in 40 mM KCl, 50 mM Tris–HCl (pH 8.3), 6 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM each of dATP, dCTP, dGTP, and dTTP, 10 units of RNase inhibitor (Promega), 100 pmol of random hexamer, total RNA and 200 units of the Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) in a final volume of 50 μL at 37° for 60 min. Polymerase chain reaction was carried out in a final volume of 20 μL, containing 1 μL of RT reaction mixture, 50 mM KCl, 20 mM Tris–HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 10 μM each of the mixed oligonucleotide primers, and 1 unit of Taq DNA polymerase (Gibco-BRL). Primers used for mouse RANKL were 5′-GACACACTACCTGACTCCTGC-3′ and 5′-CCAGTTTTCGTGC-TCCCTCC-3′ (465 bp), those for mouse OPG were 5′-ATGCCGGAGT-GTAGAGAGGAT-3′ and 5′-AAACAGCCCAGTGACCATTCCT-3′ (533 bp) [19], those for mouse RANK were 5′-GCTTGCTGCATAAA-GTCTGT-3′ and 5′-ACGTCCTAGAATCTCTGACT-3′ (708 bp); those for mouse c-fms were 5′-AACAAGTTCTACAAACTGGTGAAGG-3′ and 5′-GAAGCCTGTAGTCTAAGCATCTGTC-3′ (752 bp); those for mouse calcitonin receptor were 5′-AGAATTCCTGCATCCACCTA-3′ and 5′-TGAAAGCGTTGCACAGAGTA-3′ (558 bp) (cited from GenBank™ sequence data base), and those for β-actin were 5′-TTCTACAATGAGCTGCGTGTGGC-3′ and 5′-CTC-(A/G)TAGCTCT-TCTCCAGGGAGGA-3′ (456 bp), as reported by Waki *et al.* [20].

2.8. Data analysis

The data were analyzed using Student's *t*-test to compare the unpaired means of two sets of data. A P value of 0.05 was taken to indicate a significant difference between sets of data.

3. Results

3.1. Effects of PDE inhibitors in BMC culture

The BMCs were cultured with each PDE inhibitor (0.1–10 μM) for 7 days, and then the culture was stained for TRAP. As shown in Fig. 1, a non-selective PDE inhibitor, IBMX, and two PDE4 inhibitors with quite different chemical structures, XT-611 and rolipram, decreased the number of TRAP-positive MNCs in a concentration-dependent manner. A PDE3 inhibitor, milrinone, and a PDE5 inhibitor, zaprinast, were ineffective even at higher concentrations.

3.2. Effect of XT-611 in co-culture of ST2 cells and ACD-BMCs

Figure 2 shows the effect of XT-611 (0.1–10 μM) on TRAP-positive MNC formation in the co-culture of ST2 cells and ACD-BMCs. XT-611 significantly decreased the number of TRAP-positive MNCs in a concentration-dependent manner.

3.3. Effect of XT-611 in culture of ACD-BMCs alone

Mouse ACD-BMCs were treated with XT-611 (0.1–10 μM) and/or PGE_2 (1 ng/mL) in the presence of 25 ng/mL of M-CSF and 100 ng/mL of sRANKL for 5 days, and the number of TRAP-positive MNCs was counted. As shown in Fig. 3, XT-611 alone had little effect on the number of TRAP-positive MNCs, compared with the untreated control. PGE_2 alone slightly inhibited the MNC formation, while combined treatment with XT-611 and PGE_2 significantly decreased TRAP-positive MNC formation. Figure 4 shows the morphology of TRAP-stained cells after treatment with XT-611 and/or PGE_2.

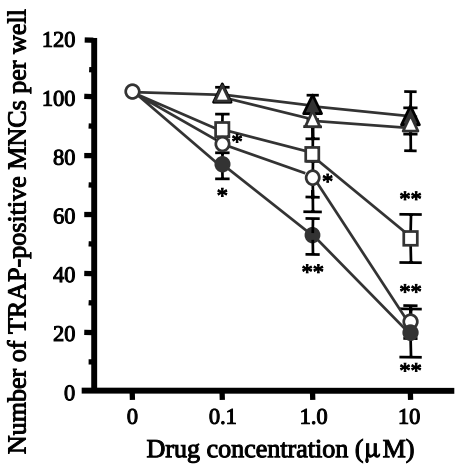

Fig. 1. Effects of PDE inhibitors on the formation of TRAP-positive MNCs in mouse BMC culture. After culture with various concentrations of each inhibitor for 7 days, cells were fixed and stained for TRAP. Each value is the mean ± SD of six experiments. (□), IBMX; (▲), milrinone; (○), rolipram; (●), XT-611; (△), zaprinast. (*, **): Significantly different from the control culture at $P < 0.05$ and <0.01, respectively.

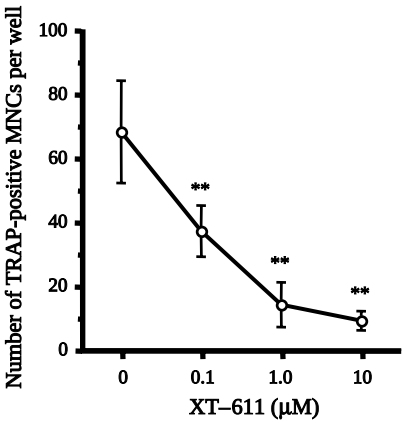

Fig. 2. Effect of XT-611 on the formation of TRAP-positive MNCs in co-culture of ST2 cells and ACD-BMCs. ACD-BMCs were cultured with ST2 cells in the presence of 10 nM dexamethasone, 10 nM 1α,25-$(OH)_2D_3$ and the indicated concentrations of XT-611 for 15 days. After the culture, cells were fixed and stained for TRAP. Each value is the mean ± SD of six experiments. (**): Significantly different from the control culture at $P < 0.01$.

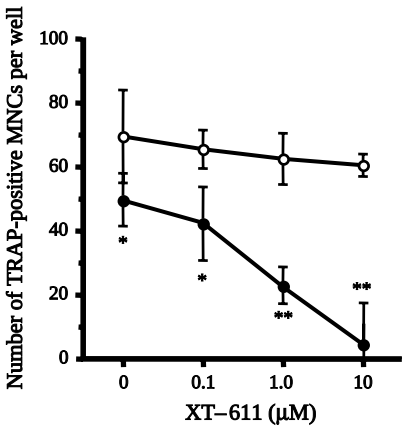

Fig. 3. Combined effect of XT-611 and PGE_2 on the formation of TRAP-positive MNCs in ACD-BMC culture. Cells were cultured with 25 ng/mL of M-CSF, 100 ng/mL of sRANKL and the indicated concentrations of XT-611 in the absence (○) or presence of 1 ng/mL of PGE_2 (●) for 5 days. After the culture, cells were fixed and stained for TRAP. Each value is the mean ± SD of six experiments. (*, **): Significantly different from each control culture at $P < 0.05$ and <0.01, respectively.

TRAP-positive MNCs appeared after treatment with XT-611 alone, but many TRAP-positive mononuclear cells were observed after combined treatment with XT-611 and PGE_2. The DNA contents in these cultures were not changed by treatment with these agents (data not shown).

3.4. Effect of XT-611 on PGE_2 production

Table 1 shows cumulative PGE_2 production by ST2 cells and ACD-BMCs for 5 days in the presence or absence of XT-611 (10 μM). ST2 cells secreted a large amount of

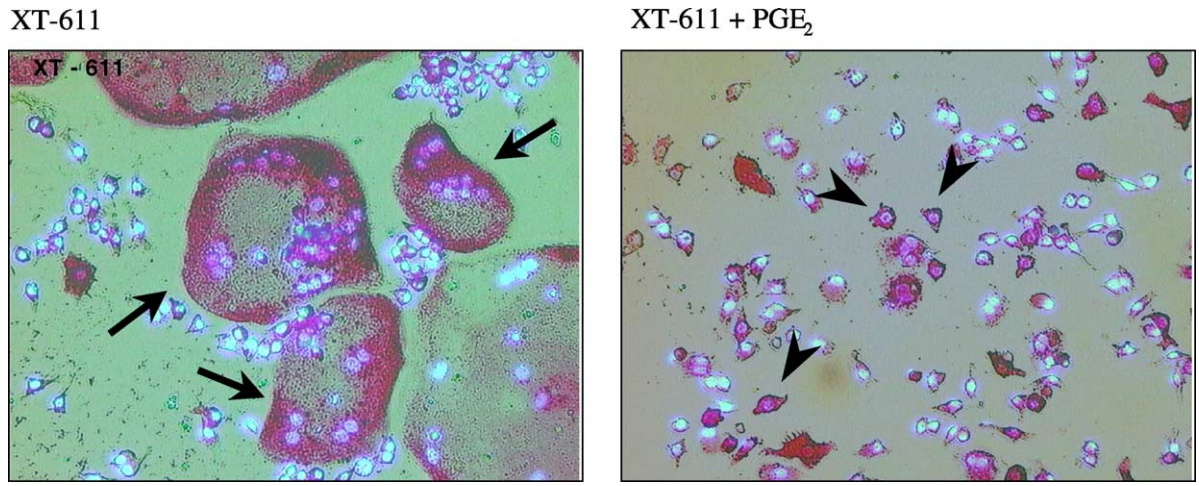

Fig. 4. Phase contrast micrographs of mouse ACD-BMC cultures. Cells were cultured with 25 ng/mL of M-CSF, 100 ng/mL of sRANKL and 10 μM XT-611 and/or 1 ng/mL of PGE_2 for 5 days. After the culture, cells were fixed and stained for TRAP and nuclei. Arrows indicate TRAP-positive MNCs. Arrowheads indicate mononuclear TRAP-positive cells (100×).

Table 1
PGE_2 production in ST2 cell culture and ACD-BMC culture

	ST2 cells		ACD-BMCs	
	Control	With XT-611	Control	With XT-611
PGE_2 (pg/mL/well)	604 ± 81	1065 ± 127**	56.4 ± 14.2	56.1 ± 15.4

ST2 cells (5×10^3 cells/well) were incubated with 10 nM 1α,25-$(OH)_2D_3$ and 10 nM dexamethasone and ACD-BMCs (4×10^5 cells/well) were incubated with 25 ng/mL of M-CSF and 100 ng/mL of sRANKL, in the presence or absence of 10 μM XT-611. After culture for 5 days, PGE_2 concentration in the medium of each well was measured. Each value represents the mean of five or six wells and the SE.

** $P < 0.01$ compared to the control.

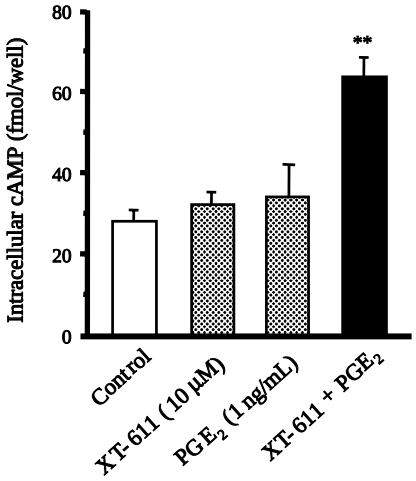

Fig. 5. Effect of XT-611 and PGE_2 on intracellular cAMP production in ACD-BMC culture. Cells were cultured for 3 days, then XT-611 (10 μM) and/or PGE_2 (1 ng/mL) were added, and the concentration of intracellular cAMP in each well was measured after 1 hr. Each value is the mean ± SD of six experiments. (**): Significantly different from the control culture at $P < 0.01$.

PGE_2 and XT-611 further increased the PGE_2 production by the cells, while in ACD-BMCs the PGE_2 production was much less than that in ST2 cells, and was not influenced by XT-611.

3.5. Effect of XT-611 and PGE_2 on intracellular cAMP in ACD-BMC culture

XT-611 (10 μM) and/or PGE_2 (1 ng/mL) were added to ACD-BMCs, and the intracellular cAMP concentration was measured for 1 hr. Figure 5 shows that the intracellular cAMP concentration was only slightly changed after addition of XT-611 or PGE_2 alone, but it was increased significantly after combined treatment with XT-611 and PGE_2.

3.6. Analysis of mRNA expression

The expression of RANKL, OPG and RANK mRNAs was analyzed during co-culture of ST2 cells and ACD-BMCs with or without 10 μM XT-611. The expression levels of these mRNAs gradually increased during culture, but were not influenced by treatment with XT-611 alone. Figure 6A shows that expression of these mRNAs was not changed by XT-611 after treatment for 15 days. Figure 6B shows changes in the expression of RANK and c-fms (an M-CSF receptor) during culture of ACD-BMCs in the presence of sRANKL and M-CSF, and the combined effect of XT-611 (10 μM) and PGE_2 (1 ng/mL). While TRAP-positive MNC formation was significantly inhibited by treatment with XT-611 and PGE_2 for 5 days (Figs. 3 and 4), the expression of these mRNAs was not changed by these agents. Only calcitonin receptor mRNA expression was decreased by the combined treatment.

4. Discussion

In this study we attempted to clarify the action mechanism of the PDE4 inhibitor on osteoclastogenesis by using three different BMC culture systems. First, we confirmed that PDE4 inhibitors decreased osteoclast formation in BMC culture in the presence of dexamethasone and $1\alpha,25\text{-}(OH)_2D_3$ (Fig. 1), as previously reported by us [8,9]. However, because many types of cells and osteotropic factors are contained in BMC cultures, it is very difficult to clearly define the nature of the cells and/or factors with which the PDE4 inhibitor interacts. Therefore,

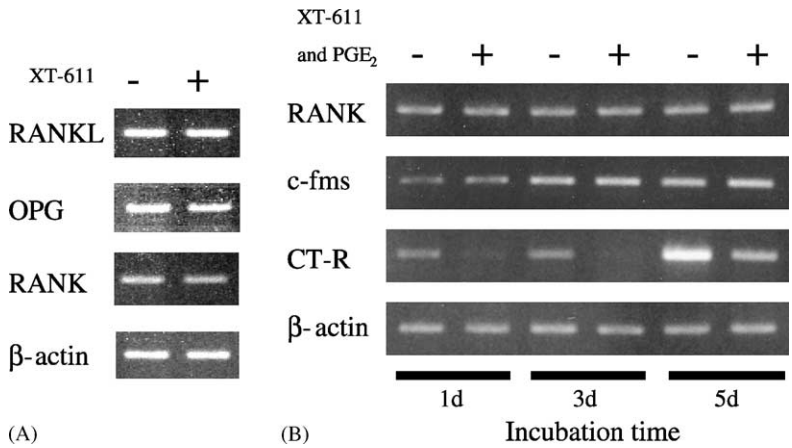

Fig. 6. Expression of mRNAs of several factors for osteoclast differentiation in co-culture with ST2 cells and ACD-BMCs (A) and in ACD-BMC-alone culture (B). (A) ST2 cells and ACD-BMCs were cultured for 15 days in the absence (−) or presence of 10 μM XT-611 (+), then expression of RANKL, OPG, RANK, and β-actin mRNAs was analyzed by a reverse transcription–polymerase chain reaction method. (B) ACD-BMCs were cultured for the indicated times in the absence (−) or presence of XT-611 (10 μM) and PGE_2 (1 ng/mL) (+), and expression of RANK, c-fms, calcitonin receptor (CT-R), and β-actin mRNAs was analyzed by a reverse transcription–polymerase chain reaction method. These experiments were done four times, and standard results were represented.

a simple co-culture system using mouse splenic cells or hematopoietic stem cells and a mouse bone-marrow stromal cell line ST2 has been established and used to study cell-to-cell interaction in the differentiation and maturation of osteoclasts [21,22]. We examined the effect of XT-611 on osteoclast formation in a co-culture of ST2 cells and mouse ACD-BMCs as a partially purified osteoclast progenitor. XT-611 also inhibited osteoclast formation in this co-culture system (Fig. 2). Finally, we examined the effect of XT-611 on osteoclastogenesis in a culture of ACD-BMCs in the presence of M-CSF and sRANKL, and found that XT-611 showed a limited effect on osteoclastogenesis but significantly inhibited in combination with PGE_2 (Fig. 3).

Some investigators have reported that PTH stimulates osteoclast formation through suppression of OPG expression in stromal cells [23,24]. In this study, however, XT-611 did not influence RANKL mRNA or OPG mRNA expression in ST2 cells. It also did not change the expression levels of RANK and c-fms mRNAs, though it did change that of calcitonin receptor mRNA, in ACD-BMCs, in combination with PGE_2 (Fig. 6). It is clear that XT-611 did not affect the expression of factors related to osteoclastogenesis. These results suggest that the inhibitory effect of XT-611 on osteoclastogenesis is not based upon a decrease in the RANK/RANKL-dependent cell-to-cell interaction, or changes in OPG production or the acceptor system for M-CSF.

After treatment with XT-611, the expression of osteotropic cytokines such as IL-1β, IL-11, and TNF-α was not changed (data not shown), but PGE_2 production was significantly increased in a culture of ST2 cells (Table 1). ACD-BMCs or mature osteoclasts secreted only a little PGE_2. Therefore, XT-611 seems to act synergistically with PGE_2, which is secreted from osteoblastic cells or stromal cells, on osteoclast progenitor cells. Indeed, XT-611 significantly inhibited osteoclast formation in the presence of PGE_2, accompanied with a marked increase of intracellular cAMP, in the osteoclast progenitor-alone culture system (Figs. 3 and 5). PGE_2 is well known to be an important local factor in regulating bone formation and resorption, and its effects are mediated by EP1, EP2, EP3, and EP4 receptors [25]. Several investigators have reported that PGE_2 stimulates the EP4 receptor on stromal cells and increases osteoclast formation in a BMC culture in the absence of active Vitamin D_3 [26,27]. Others have reported an inhibitory effect of PGE_2 on osteoclastic bone resorption and osteoclast differentiation through stimulation of EP4 receptor and an increase in cAMP in osteoclastic cells [28,29]. Roodman [30] noted that the effects of prostaglandins are dependent not only on dose, but also on the nature of the stromal cells used. In this study using ACD-BMCs, although the formation of TRAP-positive MNCs was only slightly inhibited or was not influenced by XT-611 (0.1–10 μM) or PGE_2 at 1 ng/mL, which is similar to the concentration produced by ST2 cells (Table 1), many TRAP-positive mononuclear cells rather than TRAP-positive MNCs were observed after the combined treatment (Figs. 3 and 4). The expression of calcitonin receptor, an osteoclast maturation marker [31,32], was also decreased by the combined treatment (Fig. 6). These results suggest that XT-611 and PGE_2 inhibit multinucleation or maturation of osteoclasts through a synergistic increase in cAMP in osteoclast progenitors.

XT-611 increased PGE_2 production from ST2 cells (Table 1). This may be due to an increase of intracellular cAMP by the PDE4 inhibitor through inhibition of hydrolysis of cAMP produced in cells stimulated by PGE_2 in an autocrine manner, as it has been reported that cAMP-increasing agents and protein kinase activators enhance PG production [33,34].

In conclusion, we examined the effect of XT-611 on osteoclast formation using three different BMC culture systems and found that this PDE4 inhibitor inhibits osteoclast multinucleation or maturation by acting synergistically on osteoclast progenitors with PGE_2 secreted from stromal cells, but not by influencing the cell-to-cell interaction between stromal cells and osteoclast progenitors.

On the other hand, it has been reported that PDE4 inhibitors show some adverse effects such as central nervous system stimulation and emetic action, which could not be separate from substantial PDE4 inhibitory activity, and limit their therapeutic potential [35–37]. XT-611 has been developed as a potent PDE4 inhibitor without causing emesis and other central nervous system adverse effects [16]. Therefore, this compound is considered to be promising candidates for anti-osteoporosis drugs.

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