Effects of XT-44, a Phosphodiesterase 4 Inhibitor, in Osteoblastgenesis and Osteoclastgenesis in Culture and Its Therapeutic Effects in Rat Osteopenia Models

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ABSTRACT—We have reported that denbufylline, a phosphodiesterase 4 (PDE4) inhibitor, inhibits bone loss in Walker256/S tumor-bearing rats, suggesting therapeutic potentiality of a PDE4 inhibitor in osteopenia. In the present study, effects of a new PDE4 inhibitor, 1-*n*-butyl-3-*n*-propylxanthine (XT-44), in bone were evaluated in cell cultures and animal experiments. In rat bone marrow culture, XT-44 stimulated mineralized-nodule formation, whereas it inhibited osteoclast-like cell formation in mouse bone marrow culture. In Walker256/S-bearing rats (6-week-old female Wistar Imamichi rats), rapid decrease in bone mineral density (BMD) was prominent, and oral administration of XT-44 (0.3 mg/kg, every 2 days) inhibited the decrease in BMD. In the second animal experiment, female Wistar rats (6-week-old) were sciatic neurectomized, and XT-44 was orally administered to these rats every 2 days for 4 weeks. XT-44 administration (0.3 mg/kg) recovered BMD in these neurectomized animals. Furthermore, 19-week-old, female Wistar rats were ovariectomized (OVX), and 15 weeks after surgery, these rats were orally administered XT-44 every 2 days for 8 weeks. XT-44 treatment (1 mg/kg) increased the BMD of OVX rats. These results indicate that XT-44 could be a candidate as a therapeutic drug for treating osteopenia including osteoporosis.

Keywords: Phosphodiesterase 4, Inhibitor, Walker256 carcinosarcoma, Sciatic neurectomized rat, Ovariectomized rat

Osteoporosis is characterized by low bone mass and the loss of skeletal architecture, which cause bone fracture. Since a large number of postmenopausal-aged women suffer from osteoporosis, and the number of the patients are increasing with the increase in the aged population, prevention and/or treatment for this disease is a great concern. Negative imbalance in bone remodeling, more bone-resorption than formation, elicits osteopenic disorders. Thus, antiresorptive agents, such as estrogen, calcitonin or bisphosphonate, effectively inhibit osteoclast-mediated bone resorption, and they are clinically used in the treatment for osteoporosis (1, 2). Besides, agents that stimulate osteoblast-mediated bone formation are also effective, probably being more advantageous in developed osteoporosis. There are several agents stimulating osteo-

blast growth and development. Of these, parathyroid hormone (PTH) and prostaglandin E₂ (PGE₂) have been extensively investigated. These two agents exert anabolic effects in the bone of experimental animals and also humans, although their effects depend on the method and dose of administration (3, 4). Receptors of these two agents are coupled to a variety of G-protein subunits, each of which activates its own second messenger signaling cascades; cAMP, Ca and inositol triphosphate are signaling molecules of these two agents (5, 6). Thus, some agents that affect these signal transduction pathways could exert some effects in bone tissue.

On the other hand, various phosphodiesterase (PDE) isozymes that degrade cyclic nucleotides, such as cAMP or cGMP, were characterized, and their specific inhibitors have been developed (7, 8). We have also developed 1-*n*-butyl-3-*n*-propylxanthine (XT-44), which is a PDE4 inhi-

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bitor specifically inhibiting cAMP degradation (9). We speculated that PDE inhibitors could modulate bone cell functions and could be potentially useful for the treatment of various bone diseases. Indeed, we have recently reported that denbufylline, a PDE4 inhibitor, inhibits bone loss in Walker256/S-bearing rats, which suggests the therapeutic potentiality of a PDE4 inhibitor in osteopenia (10). In the present study, first we evaluated various PDE inhibitors using rat and mouse bone marrow cultures in which bone-like mineralized nodules and osteoclast-like cells are formed, respectively. Among the PDE inhibitors examined, PDE4 inhibitors stimulated mineralized-nodule formation and inhibited osteoclast-like cell formation. Then, we evaluated the therapeutic effects of XT-44 in three different osteopenia models.

MATERIALS AND METHODS

Wistar rats and ddy mice were purchased from Sankyo Labo Service, Tokyo. Female Wistar Imamichi rats were obtained from Imamichi Institute for Animal Reproduction, Ibaraki. α -MEM was purchased from Gibco Laboratories, Grand Island, NY, USA. Fetal bovine serum was purchased from Summit, Collins, CO, USA. Culture plates were obtained from Sumitomo, Tokyo. The phosphate ester of ascorbic acid was purchased from Wako Chemical Co., Osaka. β -Glycerophosphate, dexamethasone and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Chemical Co., St. Louis, MO, USA. 1,25(OH)₂ vitamin D₃ was a gift from Teijin Pharmaceutical Co., Tokyo. Milrinone and zaprinast were purchased from Calbiochem-Novabiochem Intl., La Jolla, CA, USA. XT-44 (11) and rolipram (12) were synthesized in our laboratory, according to the reported methods. All the following experiments were performed according to the Animal Experiment Guideline in Tokyo Medical and Dental University.

Rat bone marrow cell culture for osteoblastic differentiation

Rat bone marrow cells were prepared from male Wistar rats (5-week-old) according to the method of Maniopoulos et al. (13) with some modifications as previously described (14, 15). Bone marrow cell suspension was inoculated into 12-well plates at a cell density of 10^6 cells/cm² and then cultured. The culture medium was α -MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 60 μ g/ml kanamycin. The cells were maintained at 37°C in a humidified atmosphere consisting of 95% air and 5% CO₂. After 24 hr, the entire medium and floating cells were removed by aspiration and changed to a medium that was supplemented with 0.2 mM phosphate-ester of ascorbic acid, 1 mM β -

glycerophosphate and 10 nM dexamethasone. The medium was changed every two days. The cells were treated with PDE inhibitors at day 3 for 24 hr. At day 14, the cultures were fixed with methanol and stained with Alizarin Red S. The stained image was acquired with a densitometer (GS-670, Bio-Rad) and analyzed with software (NIH image 1.59) in a Macintosh computer.

Mouse bone marrow culture for osteoclast formation

Mouse bone marrow cells were prepared from femurs and tibiae of 8-week-old male ddy mice and cultured as previously described (16) with some modifications (17). The whole bone marrow cells were suspended, counted and then centrifuged as described above. The cell suspension was inoculated into 24-well plates at a cell density of 10^6 cells/well and then cultured. Medium was α -MEM supplemented with 10% heat-inactivated fetal bovine serum, 60 μ g/ml kanamycin and 10 nM 1,25(OH)₂ vitamin D₃. The cells were treated with PDE inhibitors from day 1 to day 7. At day 7, cultures were fixed with 10% formalin and stained with tartrate-resistant acid phosphatase (TRAP) in sodium acetate buffer (pH 5.0) containing naphthol AS-MX phosphate, red violet LB salt and 50 mM sodium tartrate. Cells that are TRAP-positive and containing three or more nuclei were counted under a microscope.

Treatment with PDE inhibitors in cell culture

Various PDE inhibitors were dissolved in dimethyl sulfoxide at 10 mM as a stock solution and added to the above culture systems. The final concentration of dimethyl sulfoxide did not exceed 0.1%, which affected neither mineralized-nodule formation in rat bone marrow cell culture nor osteoclast-like cell formation in mouse bone marrow cell culture.

XT-44 administration in Walker256/S-bearing rats

Walker256/S carcinosarcoma was maintained by serial (2-week interval) subcutaneous transplantation in 6-week-old, female Wistar Imamichi rats. Thirty female rats were divided into 5 groups. Twenty-four rats of 4 groups were subcutaneously inoculated in the back with 1 mm³ of the carcinoma from donors. XT-44 was suspended in 0.5% CMC solution. One day after transplantation, the rats of three tumor-bearing groups were orally administered with XT-44 at the doses of 0.3, 1 and 3 mg/kg. Six rats without tumor transplantation and 6 tumor-bearing rats were administered with vehicle. Administration was every 2 days for 13 days, and the animals were sacrificed 14 days after transplantation.

XT-44 administration in sciatic neurectomized (SN) rats

A total of 30 female Wistar rats (6-week-old) were ran-

domly divided into 5 groups. Twenty-four rats of 4 groups were sciatic neurectomized (SN), and 6 rats of one group were sham-operated (SHAM). One day after neurectomy, the rats of three SN groups were orally administered with XT-44 at doses of 0.3, 1 and 3 mg/kg. Six rats of the SHAM group and 6 rats of the SN group were administered with vehicle. Administration was every 2 days for 4 weeks and the animals were sacrificed 4 weeks after surgical operation.

XT-44 administration in ovariectomized (OVX) rats

A total of 30 female Wistar rats were randomly divided into 5 groups. Twenty-four rats of 4 groups were ovariectomized (OVX) and 6 rats of one group were SHAM at the age of 19 weeks. During the experiment, the rats were pair-fed. Fifteen weeks after the operations, three OVX groups were orally administered with XT-44 at doses of 1, 3 and 10 mg/kg. The SHAM group and one OVX group were administered with vehicle. Administration was every two days for 8 weeks and animals were sacrificed 23 weeks after the operation. This experimental schedule is to examine the treatment effect rather than the prevention effect because drug administration started 15 weeks after ovariectomy, not soon after the surgical operation. The animals of this experiment were older than the ones of the above two experiments and exerting therapeutic effects in this experimental schedule seemed more difficult than in the usual prevention model in OVX rats. Thus, we decided to increase doses of XT-44 in this experiment.

Measurement of bone mineral density (BMD)

BMD of whole femurs was measured by dual energy x-ray absorptometry (DEXA) using DCS-600R (Aloka Co., Tokyo), which was modified for small animals based on DCS-600, a standard DEXA model to measure human radius BMD.

Statistical analyses

All values are represented as the mean $\pm$ S.D. Statistical significance was determined by the unpaired Student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

In rat bone marrow culture, treatment with rolipram or XT-44 stimulated mineralized-nodule formation in a concentration-dependent manner, whereas IBMX and zaprinast also stimulated mineralized-nodule formation (Fig. 1A). However, a higher concentration of zaprinast was necessary for this stimulation, and milrinone did not show any such effect (Fig. 1A). In mouse bone marrow culture, XT-44, rolipram and IBMX inhibited TRAP-

Fig. 1. Effects of PDE inhibitors on mineralized-nodule formation in rat bone marrow culture (A) and on TRAP-positive multi-nucleated cell formation in mouse bone marrow culture (B). The picture in panel A presents a stained image of mineralized-nodules after treatment with different concentrations of XT-44. In the picture, dark black dots are mineralized-nodules, and the concentration of XT-44 are presented above the picture. XT-44 (●), IBMX (○), rolipram (▲), milrinone (△) and zaprinast (□). In the control culture, cells were not treated with any PDE inhibitor, cultured and evaluated at day 14 for mineralized-nodule formation (A) and at day 7 for TRAP-positive multi-nucleated cell formation (B). The number of the mineralized nodules and the number of TRAP-positive multi-nucleated cells in the control cultures were 48.6 ± 8.3 ($n=12$) and 121 ± 17 ($n=10$), respectively. Data are converted to percent of control and represented as the mean $\pm$ S.D. ($n=4$). ****Significantly different from the control culture at $P < 0.001$.

Fig. 2. Effect of XT-44 on femur BMD of Walker256/S-bearing rats. The indicated doses of XT-44 were orally administered to Walker256/S-bearing rats every 2 days for 13 days, and the total BMD of right femurs was measured at day 14 after the tumor inoculation. The control group was intact animals that were not transplanted with Walker256/S. Data are represented as the mean $\pm$ S.D. (n=6). **, ***, **** Significantly different from the Walker256/S-bearing rats administered with vehicle at $P < 0.01$, $P < 0.005$ and $P < 0.001$, respectively.

positive multi-nucleated cell formation in a concentration-dependent manner, and neither milrinone nor zaprinast showed an inhibitory effect (Fig. 1B).

In our preliminary experiment, the body weight of rats decreased slightly when we administered XT-44 at a dose of 10 mg/kg every day. However, XT-44 administration at a dose of 10 mg/kg every 2 days did not cause any difference of body weight among the administered and not-administered groups. Thus, we decided to use XT-44 administration every 2 days in the present study. In Walker256/S-bearing rats, decrease in femoral BMD was observed 2 weeks after the tumor inoculation and oral administration of XT-44 at dose of 0.3–3 mg/kg every 2 days inhibited this BMD decrease in these tumor-bearing animals (Fig. 2). Four weeks after neurectomy, femoral BMD of SN rats was less than that of SHAM rats, and XT-44 administration at a dose of 0.3–3 mg/kg for 4 weeks after surgery recovered the BMD level of SN rats (Fig. 3). Femoral BMD of OVX rats was less than that of SHAM rats 23 weeks after ovariectomy; however, XT-44 administration at dose of 3–10 mg/kg for 8 weeks from 15 weeks to 23 weeks after ovariectomy increased BMD level (Fig. 4).

Fig. 3. Effects of XT-44 on femur BMD of sciatic neurectomized (SN) rats. The indicated doses of XT-44 were orally administered to SN rats every 2 days for 4 weeks, and total BMDs of right femurs were measured at 4 weeks after the operation. Data are represented as the mean $\pm$ S.D. (n=6). **, ***, **** Significantly different from the SN rats administered with vehicle at $P < 0.01$, $P < 0.005$ and $P < 0.001$, respectively.

DISCUSSION

In rat bone marrow culture, some of the stromal cells differentiate to osteoblasts and subsequently bone-like mineralized-nodules are formed. In this process, osteoblastic phenotypes such as alkalinephosphatase (ALP) activity and matrix protein expression are time-dependently regulated (15). Using this culture system, we have evaluated biocompatibility of biomaterials, which are applied to bone tissue, by measuring osteoblastic phenotype expression (ALP activity and mRNA expression of bone matrix proteins) and mineralized-nodules (18, 19). Based on the results of these studies, we assume that the amount of mineralized-nodules well reflects the biocompatibility of the biomaterials. This speculation is reasonable because expression levels of osteoblastic phenotypes are time-dependent, whereas the amount of mineralized-nodules is considered to be a final accumulative result of sequential osteoblastic phenotype expression. Thus, we evaluated effects of PDE inhibitors by measuring mineralized-nodule in the present study.

We have observed that PGE₂ or PTH stimulates mineralized-nodule formation when cells were treated

Fig. 4. Effects of XT-44 on femur BMD of ovariectomized (OVX) rats. Female rats were OVX or SHAM-operated at the age of 19 weeks. XT-44 administration was started 15 weeks after the operation. The indicated doses of XT-44 were orally administered to OVX rats every 2 days for 8 weeks, and total BMDs of right femurs were measured at 23 weeks after the operation. Data are represented as the mean $\pm$ S.D. ($n=6$). **, ***, ****Significantly different from the OVX rats administered with vehicle at $P<0.01$, $P<0.005$ and $P<0.001$, respectively.

with these agents at an early culture stage; i.e., at day 3 or at day 4 in culture (20, 21). Thus, we treated cells with PDE inhibitors at day 3. Although IBMX is a PDE inhibitor, its specificity to PDE isozymes is low, whereas XT-44 and rolipram are specific PDE4 inhibitors (9, 22). These three compounds rather than milrinone (a PDE3 inhibitor) (23) and zaprinast (a PDE5 inhibitor) (24) stimulated mineralized-nodule formation. Although zaprinast stimulated mineralized-nodule formation, a higher concentration (10 μ M) was necessary to exert these effects and the level of the stimulation at this concentration was not high. Notably, XT-44 and rolipram exerted these stimulative effects at much lower concentrations of 0.1 and 1 μ M, respectively. These concentrations of XT-44 and rolipram were comparable to the 50% inhibitory concentrations of these two compounds for PDE4 inhibition in guinea pig trachea (9, 22). Since the PDE4 inhibitor specifically inhibits cAMP degradation, a cAMP increase is likely to be mainly implicated in this anabolic effect in rat bone marrow culture. Our previous finding that dibutyryl-cAMP also exerts a similar anabolic effect in this culture system supports this speculation (10). We have recently observed that rolipram treatment increases

the number of mineralized-nodules in this culture system without stimulation of cellular proliferation; furthermore, when bone marrow cells were prepared from the rats treated with rolipram, these cells produce more mineralized-nodules than the cells from untreated rats (25). Since the number of mineralized-nodules corresponds with the osteoprogenitor cell population (26), it is assumed that PDE4 inhibitors including XT-44 could stimulate osteoblastic differentiation not only in culture but also in vivo.

In the present study, IBMX, XT-44 and rolipram inhibited osteoclast-like cell formation in mouse bone marrow culture. PDE4 inhibitors exerted more remarkable inhibitory effects than PDE3 and PDE5 inhibitors in the present study, and we have observed that dibutyryl-cAMP also inhibits osteoclast-like cell formation in this culture system (10). These evidence indicate that an agent that increases cAMP could inhibit osteoclast-like cell formation in this culture system.

Although which phosphodiesterase isozymes exist in bone cells has not been clarified yet, our results in cell culture suggest that a PDE4 inhibitor could be a signal modulator in bone. Acting on osteoblastic and/or osteoclastic differentiation and function of these cells, a PDE4 inhibitor could change the balance between bone resorption and formation, although the underlying mechanism of our findings in cell culture is not clear. To examine the therapeutic potentiality of XT-44 in osteopenia, we proceeded to animal experiments. We used three osteopenia models; however, the mechanism of bone loss in each model is different.

In the first model, Walker256/S-bearing rat, there is a pronounced decrease in the serum 17β -estradiol level and rapid BMD decrease (27). We think this model is advantageous for evaluating the therapeutic effect of a drug in short time period, and we have reported that calcitonin administration effectively inhibits bone loss in this model (27). In addition to the decrease in the serum 17β -estradiol level, administration of 17β -estradiol inhibits this bone loss (27), suggesting that hypoovarianism underlies this osteopenia. Furthermore, we have recently observed that the serum tumor necrosis factor (TNF)- α level is remarkably high in this model (28). TNF- α stimulates osteoclast formation and its activation (29, 30), whereas this factor also induces apoptosis of osteoblasts (31–33). In this osteopenia model, we have observed an increase in osteoclast number and decrease in osteoblast number (10), which corresponds with the elevation of TNF- α .

The second osteopenia model, sciatic neurectomized rat, is a model of bone loss under less mechanical stress. It is well known that bone mass and structure are well controlled by mechanical stress; however, the mechanism of this phenomenon has not been clarified, although

PGE₂ and nitric oxide are possible mediators controlling osteoblastic function under mechanical stress (34–36). Decrease in bone formation rather than increase in bone resorption is characteristic after sciatic neurectomy (37).

In the final animal experiment, OVX rats were used, which mimics bone loss in the estrogen deficiency condition of the postmenopausal woman. Ovariectomy induces an increase in bone turnover, more increase in bone resorption than bone formation, resulting in the reduction of bone mass (38), and implication of interleukin (IL)-1, IL-6 and TNF- α in this bone loss has been strongly suggested (39, 40).

In the present study, our PDE4 inhibitor XT-44 exerted a therapeutic effect in all three osteopenia models. Based on the present results in cell culture, stimulation of osteoblastic differentiation and inhibition of osteoclastic differentiation are a conceivable mechanism of XT-44 therapeutic effects in these three osteopenia models. Furthermore, suppression of cytokine production by XT-44 is also likely in both Walker256/S-bearing rats and OVX rats. In these two osteopenia models, TNF- α is a key cytokine that causes bone loss. In Walker256/S-bearing rats, we have observed a decrease in osteoclast number and increase in osteoblast number when the tumor bearing rats are treated with a PDE4 inhibitor, denbufylline (10). There is accumulating evidence that PDE4 inhibitors suppress TNF- α production (41, 42). Although we did not measure the TNF- α level in the present experiment, suppression of TNF- α is one possible mechanism of the XT-44 therapeutic effect. In addition to TNF- α , PDE4 inhibitors also suppress IL-1 and IL-6 production (42, 43). TNF- α , IL-1 and IL-6 are most likely key cytokines in bone loss in estrogen-deficient conditions (39, 40). Thus, suppression of the productions of these three cytokines could be an explanation for the therapeutic effect of XT-44 in OVX rats.

In the present study, XT-44, a PDE4 inhibitor, stimulated osteoblastic differentiation and inhibited osteoclastic differentiation in murine bone marrow culture. Furthermore, oral administration of XT-44 exerted therapeutic effects in three osteopenia models. The present results indicate that XT-44 could be a candidate as a therapeutic drug for treating osteopenia including osteoporosis. The mechanism of XT-44 therapeutic effects in these three osteopenia models, especially whether XT-44 stimulates bone formation and/or inhibits bone resorption, should be clarified in histomorphometric studies in the future.

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