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Prostaglandin E EP4 Receptor Agonist Induces the Bone Formation by an Alteration of the Osteoblast and Osteoclast Dynamic State

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Abstract: To investigate the mechanism of bone formation by EP4 activation, an osmotic pump was implanted subcutaneously into the backs of rats and an EP4 receptor agonist was administered at a dose of 100 ng/kg/min for up to 28 days. The histology of the femur (including bone marrow) and the serum and/or urinary bone metabolism parameters were examined on Day 0, 1, 3, 5, 7, 14, and Day 28. In EP4 receptor agonist treated-rats, increase of osteoclasts in the metaphysis was observed on Day 1 and the number of osteoblast showed an increase from Day 3. In addition, cancellous bone and endosteal bone formation were observed in the metaphysis and diaphysis from Day 5 and this peaked on Day 28. Serum alkaline phosphatase activity showed a transient decrease on Day 1, but thereafter showed an increase. The Gla-type osteocalcin level showed an increase from Day 1. Moreover, Gla/Glu osteocalcin ratio showed an increase on Day 5. The urinary excretion of deoxypyridinoline increased on Day 3, showed a transient decrease on Day 5, and increased once again from Day 7. These results indicate that EP4 receptor agonist-induced bone formation is related to an increase of osteoclasts at the initial stage and a subsequent increase of osteoblasts.

Key words: PG E EP4 receptor agonist, osteoblast, osteoclast, adipocyte, rat

Introduction

Prostaglandins (PGs) are a group of lipid mediators that are produced from arachidonic acid in a variety of tissues under various physiological and pathophysiological conditions and act to maintain local homeostasis. It is also well known that PGs mediate inflammatory reactions1. On the other hand, many investigators have demonstrated that administration of PGE1 and PGE2 can affect bone formation and/or bone resorption in animals and humans2–8. Namely, PGs are well known to be important local factors in regulating bone formation and resorption.

The PG E2 receptor has been pharmacologically identified as having four different subtypes (EP1, EP2, EP3 and EP4)1. These receptors are encoded by distinct genes and are expressed differentially in the body. Recently, Sakuma et al.9 and Miyaura et al.10 showed that EP4 receptor mediates PGE2-induced osteoclast differentiation in bone organ culture. On the other hand, Yoshida et al.11 observed that EP4 activation induced de novo bone formation within immobilized rats. We were interested in the coordination of these two EP4 actions on the bone in rats treated with EP4 receptor agonist, as well as the detailed morphological changes of the bone. But, an EP4 receptor agonist-induced serial morphological observation was not done.

In this study, we investigated the mechanism of bone formation by EP4 activation using EP4 receptor agonist treated rats. Serial examination of the femur was done by histological, histomorphometric, histochemical, and immunohistochemical techniques for up to 28 days. We also measured the changes of bone formation and bone resorption markers, such as serum osteocalcin (OC), serum alkaline phosphatase (ALP), and/or urinary deoxypyridinoline (D-pyr) excretion.

Materials and Methods

Animals

Sixty-five 7-week-old Crj: CD (SD) IGS male rats
(weighing 213.4–242.0 g) were purchased from Charles River Japan, Inc. The animals were housed in hanging stainless steel wire cages (5 per cage) in a room maintained at a temperature of 23 ± 2°C and a humidity of 55 ± 10% with lights on for 12 hours (from 8:00 to 22:00). The rats were fed a standard pellet diet (CRF-1, Oriental Yeast Industry Co. Ltd.) and tap water ad libitum. After one week of quarantine, the animals were used in this experiment. The experiments were conducted in accordance with in-house guidelines for laboratory animal experimentation.

Dosing procedures and experimental design

Five rats were sacrificed on Day 0 as the initial controls. Thirty rats were administered an EP4 receptor agonist (ONO-4819; methyl 7[(1R, 2R, 3R)-3-hydroxy-2-[(E)-(3S)-3-methoxymethylphenyl]-1-butenyl]-5-thiaheptanoate) via an osmotic pump (Alzet®, Alza Corporation, Palo Alto, CA, USA, Mean pumping rate: 9.9 µl/hr). The EP4 receptor agonist was dissolved in saline so that a dose of 100 ng/kg/min (based on the initial content) was delivered from a pump implanted into the subcutaneous tissue in the back for up to 28 days. The dosing volume was calculated on the basis of body weight and then the pump was filled with fixed concentration. The dose level was set at 100 ng/kg/min, which was sufficient to evaluate the bone formation effect of EP4 on femur bone. The remaining 30 rats received saline in the same way and were used as age-matched controls. The rats were weighted on alternate days and the osmotic pump was changed every second day. The osmotic pump was exchanged 13 times maximum. Five animals in each group were used for examination.

Table 1. Body Weight Change in the Rats Treated with EP4 Receptor Agonist for 28 Days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0†</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>250.7 ± 5.2c</td>
<td>256.1 ± 5.3</td>
<td>268.1 ± 7.4</td>
<td>280.0 ± 4.1</td>
<td>285.4 ± 6.8</td>
<td>322.4 ± 10.1</td>
<td>363.5 ± 6.1</td>
</tr>
<tr>
<td>EP4 agonistb</td>
<td>256.0 ± 4.5</td>
<td>245.4 ± 4.7</td>
<td>269.4 ± 6.6</td>
<td>285.7 ± 7.4</td>
<td>325.8 ± 10.5</td>
<td>373.1 ± 11.4</td>
<td></td>
</tr>
</tbody>
</table>

a: Initial control. b: EP4 receptor agonist (100 ng/kg/min). c: Mean ± SD (g).

Histology and histomorphometry

For the histological and histomorphometric studies, one femur (including bone marrow) was removed from each rat and fixed in 10% phosphate-buffered formalin for 48 hours. After decalcification with 2% EDTA, the specimen was embedded in paraffin. Subsequently, deparaffinized 4 µm thick longitudinal sections were cut and stained with hematoxylin and eosin (H-E) for histological and histomorphometric examination. For the histomorphometric analysis, the number of osteoclasts, osteoblasts, and adipocytes were counted in the bone marrow in H-E sections. The number of osteoclasts were counted at × 200 magnifications in all fields in the metaphysis and the diaphysis regions, while osteoblasts and adipocytes were counted at × 400 magnifications in five randomly selected fields in the metaphysis and the diaphysis regions per animal. As well, the cancellous bone and the endosteal bone area in the metaphysis and the diaphysis regions were measured as follows. In brief, slides of longitudinal sections of the femur (including bone marrow) were processed using image analysis software (Adobe Photoshop® 4.0J; Adobe Systems, San Jose, CA, USA) after being scanned into a computer via a MINOLTA Quick Scan 35 (Minolta, Osaka, Japan). After using Photo Shop to copy the femur, the endosteal bone mass area (mm²) was measured using image analysis software (NIH Image 1.61/fat; NIH, Bethesda, MD, USA).

Histochemistry and immunohistochemistry

Tartrate-resistant acid phosphatase (TRAP) and Cathepsin K were detected using deparaffinized 4 µm thick longitudinal sections. TRAP activity was detected by the method of Barka and Anderson12, using naphthol AS-BI phosphate (Sigma St. Louis, MO, USA) as the substrate, with 50 mmol/L sodium tetrathionate in the working solution. Cathepsin K immunohistochemistry was done by the avidin-biotin-peroxidase complex (ABC) method (Vectastain® Elite ABC Kit; VECTOR LAB INC). Sections were stained with anti-cathepsin K (1:500) for 1 hour at room temperature. Anti-cathepsin K antibody was kindly provided by the 1st Department of Anatomy, Faculty of Dentistry, Niigata University.

Statistical analysis

The number of osteoblasts, osteoclasts, or adipocytes,
Results

Clinical signs and body weight

No animal died in the EP4 receptor agonist-treated rats. And no abnormalities of a clinical sign were observed in the EP4 receptor agonist-treated rats. Although, body weight in the EP4 receptor agonist-treated rat showed a transient decrease on Day 3 and Day 5, but thereafter showed an increase (Table 1).

Histological findings (including histochemical and immunohistochemical analyses)

In the EP4 receptor agonist-treated rats, increase of TRAP-positive multinucleated cells on the surface of trabecular bone in the metaphysis were observed on Day 1. Cells located in the same region also showed immunolocalization of Cathepsin K, suggesting that these were osteoclast. There were numerous resorption cavities and crevices containing osteoclasts in trabecular bone (Fig. 1A–F). As well as these cancellous bone was thinned and compared with control ones. Cuboidal osteoblasts and fibroblast-like cells were also seen at the metaphysis and diaphysis from Days 3 and 5 in the EP4 receptor agonist-treated group (Fig. 1G, H), but no changes were observed in the ephyesal cartilage (Fig. 1A, C). Increased cancellous bone and endosteal bone formation were observed in the metaphysis and diaphysis from Day 5 and it peaked on Day 28 (Fig. 1J). We also confirmed that the formed endosteal bone on Day 28 by the EP4 receptor agonist becomes a positive reaction shown by von Kossa’s stain using undecalcified bone section (data not shown). Cancellous bone was thickening as compared with age-matched control.

On the other hand, the number of adipocyte around the cancellous bone and endosteal bone tissues in the marrow showed a marked decline in EP4 receptor agonist-treated rats compared with age-matched controls on Day 28 (Fig. 1I, J). The number of adipocytes increased until the end of the experiment (124.4 ± 38.3 to 235.8 ± 68.5 cells). The number of osteoblasts ranged from 39.0 ± 12.2 to 49.4 ± 19.7 cells during the experimental period.

In EP4 receptor agonist-treated rats, the number of osteoblasts showed a marked increase on Day 3 (1158.0 ± 87.8 cells) compared with Day 0 and Day 1 (434.4 ± 58.3 and 431.4 ± 68.4 cells respectively). This increase continued until the end of the experiment. In the control rats, the number of osteoblasts ranged from 397.2 ± 100.5 to 472.6 ± 48.6 cells during the experiment.

In control rats, the number of adipocytes increased throughout the experimental period and peaked on Day 28 (790.6 ± 388.0 cells). In contrast, bone marrow adipocytes decreased over time in EP4 receptor agonist-treated rats, and showed a marked decrease on Day 28 (126.0 ± 41.9 cells) compared with that in control rats.

The endosteal bone mass area of EP4 receptor agonist-treated rats showed an increase on Day 7 (1.03 ± 0.11 mm²) and peaked on Day 28 (1.32 ± 0.19 mm²). In control rats, the endosteal bone mass area ranged from 0.55 ± 0.14 to 0.71 ± 0.12 mm² during the experimental period.

Bone metabolism markers

The changes of bone metabolism markers are shown in Figs. 5 and 6.

Serum Gla-type OC as a bone formation marker showed an increase in EP4 receptor agonist-treated rats compared with control rats from Day 1 to the end of the experiment. Serum Glu type OC as a bone resorption marker showed a transient increase in EP4 receptor agonist-treated animals on Day 14. Furthermore, serum Gla/Glu OC ratio showed a significant higher value in EP4 receptor agonist-treated animals on Day 5 (p<0.01) and the high value was shown as a whole. This finding showed that bone metabolism turnover is accelerating by treated of EP4 receptor agonist. Serum ALP showed a transient decrease on Day 1 (p<0.01), and thereafter was increased in EP4 receptor agonist-treated rats (p<0.05, on Day 28). Urinary D-pyr excretion was increased on Day 3 (p<0.05), but showed a transient decrease on Day 5 in EP4 receptor agonist-treated rats, compared with age-matched control rats. Although, it showed an increase from Day 7 onwards (p<0.01).

Discussion

Repeated administration of an EP4 receptor agonist has been shown to induce new bone formation in rat. Our study revealed two interesting results. Firstly, we showed that EP4 receptor agonist-induced bone formation is characterized by an increase of osteoclasts at an early stage and a subsequent increase of osteoblasts. Yoshida et al.11 observed that administration of an EP4 receptor agonist restored bone mass and strength normally lost in rats subjected to ovariectomy or immobilization. Generally, PGE2 is considered to be a potent stimulator of bone resorption because of enhancing osteoclast formation by its indirect action through stromal cells. Nevertheless, Mano et al. showed that PGE2 directly inhibits bone-resorbing activity of functionally mature osteoclasts by activation of the adenylate cyclase system, mainly through EP413. In this
study, serial morphological changes of the femur generally corresponded with changes in the number of osteoclasts, and osteoblasts, as well as the endosteal bone mass area. In the EP4 receptor agonist treated-rats, osteoclast proliferation and activation was observed on Day 1 in cancellous bone at the metaphysis. Activated cuboidal osteoblasts and fibroblast-like cells were both observed on Day 3. Cancellous bone and endosteal bone formation at the metaphysis and/or diaphysis was also observed from Day 5 onwards and this increased as the experimental period was prolonged. Moreover, these changes showed a positive correlation with bone formation and/or resorption markers such as the serum Gla-type OC concentration and serum ALP activity (a bone formation parameters) or urinary D-pyr excretion (a bone resorption parameter). These results indicate that EP4 receptor agonist-induced bone formation is characterized by an increase of osteoclasts at an early stage and a subsequent increase of osteoblasts. Above results suggest that activation of EP4 induces bone remodeling in vivo.

Secondly, we observed that adipocytes markedly decreased by the repeated administration of an EP4 receptor agonist around the cancellous and endosteal bone tissues in the marrow. Osteoblasts and adipocytes arise from a common progenitor cell in the bone marrow14-16. In cultures of bone marrow cells from wild-type mice, PGE2 induced the expression of core-binding factor alpha-1 (Cbfa 1) and enhanced formation of mineralized nodules, both of which were absent in cultures of cells from EP4-deficient mice17. Cbfa 1 is an osteoblast-specific transcription factor that is an important determinant of osteoblast differentiation, and EP4 promotes the activation of Cbfa 1 in vitro11. On the other hand, Cbfa 1 was shown to suppress bone marrow adipocyte differentiation from marrow stromal progenitors in cultures of calvarial cells from Cbfa 1-deficient mice18. In this study, the EP4 receptor agonist caused a marked decrease of adipocytes around the bone tissues in the marrow on Day 28. The bone mass area also showed a negative correlation with

vivo.
the number of bone marrow adipocytes. Although we did not examine the expression and localization of Cbfa 1 in this study, the possibility was suggested that activation of Cbfa 1 by the EP4 receptor agonist suppressed marrow adipocyte differentiation and enhanced osteoblast formation from progenitors in the bone marrow stroma. Further studies and needed to determine the exact mechanisms underlying these phenomena.

References

Fig. 2. Number of osteoclast, osteoblast and endosteal bone area in the femur of rats given the EP4 receptor agonist for 28 days. Statistical significance was analyzed using Student’s t-test (#: p<0.05, ##, **: p<0.01, ###: p<0.001) compared with the saline control value (*** shows osteoclast, #, ### shows bone area).

Fig. 3. Number of osteoclast, osteoblast and endosteal bone area in the femur of rats given the saline for 28 days.

Fig. 4. Number of adipocyte and endosteal bone area in the femur of rats given the EP4 receptor agonist or the saline for 28 days. Statistical significance was analyzed using Student’s t-test (#: p<0.05, **: p<0.01, ###: p<0.001) compared with the saline control value (# shows an adipocyte, **, ### shows bone area).


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