Chronic Intramedullary Infusion of Prostaglandin E2 Stimulates Bone Formation Both in the Bone Marrow and in the Periosteum

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Abstract. Prostaglandin E2 (PGE2) has a potent bone resorbing activity *in vitro*, but some recent studies have shown that PGE2 stimulates bone formation *in vivo*. The effects of PGE2 on the bone are therefore still controversial. We attempted to reveal the effects of PGE2 on bone *in vivo* more directly; we injected PGE2 continuously into the bone marrow and onto the periosteum and examined the local effects of PGE2 histologically or by bone densitometry. Following PGE2 infusion into the bone marrow, new bone was formed in the bone marrow around the infused site and following PGE2 infusion onto the periosteum, extensive periosteal bone formation was observed. Bone mineral content was also increased significantly in the PGE2 infused bones. The administration of cyclic AMP did not mimic the effects of PGE2. In contrast to *in vitro* experiments, the *in vivo* effect of PGE2 is predominantly to produce bone.

Key words: Prostaglandin E2, Bone mineral content, Bone formation, Periosteal reaction
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PROSTAGLANDINS (PGs) have been shown to play an important role in bone remodeling [1]. It is well known that prostaglandin E (PGE) has a potent bone resorbing activity *in vitro*. PGE was a candidate for bone resorbing factors in rheumatoid arthritis, cancer-mediated hypercalcemia and osteolysis of bone metastasis, but several lines of evidence show that PGE stimulates bone formation *in vivo*: the systemic administration of prostaglandins, such as the daily subcutaneous injection of prostaglandin E2 (PGE2) [2–10] and a slow release pellet containing prostaglandin D2 [11], stimulated bone formation. The role of prostaglandins on the bone therefore remains to be clarified. To reveal the *in vivo* effects of PGE2 on the bone more directly, we used the method in which PGE2 is continuously injected into the bone

marrow and onto the periosteum in rats by means of osmotic mini-pumps, and examined the effects of PGE2 histologically and in bone mineral measurements. Our results confirmed an anabolic effect of PGE2 on bone *in vivo*.

Material and Methods

Materials

PGE2 and 8-bromo-cAMP (8-B-cAMP) were purchased from Sasaki Chemical Co., Ltd. (Kyoto, Japan). PGE2 was dissolved in ethanol and diluted in phosphate buffered saline (PBS; pH 7.4) with 1% heat-inactivated bovine serum albumin (dilution buffer) for use. 8-bromo-cAMP was diluted in dilution buffer for use.

Infusion in Rats

10–12 month-old female Wistar rats (SPF; 270–

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290 g) were obtained from Shimizu Animal Supply Co. (Kyoto, Japan) and housed in light- and temperature-controlled rooms (20–24 °C and 12 h/12h light-dark cycle) under sanitary conditions. During the experiment periods, each animal was housed in a separate plastic cage and allowed free access to water and a 0.5% calcium diet. The animals were maintained under the guidelines established by the Animal Care Advisory Committee of Shiga University of Medical Science. PGE2 (10 to 100 µg) or vehicle alone was dissolved in 10 µl of 90% ethanol and diluted in dilution buffer and infused into the left femur with an Alzet mini-osmotic pump (model 2002, the mean filling volume was 213 µl; Alza, Palo Alto, CA). In case of intramedullary infusion, a hole 0.9 mm in diameter was made in the left femoral shaft with a motor-driven drill, and the 3 mm long tip of a 21 gauge stainless steel needle was inserted in such a way that the tip of needle was located in the center of the bone marrow. In the case of infusion onto the periosteum, the 5 mm long tip of a 21 gauge stainless steel needle was attached to the periosteum of the mid-femoral shaft. The needle tip was fixed to the bone with a fine thread and connected to the mini-pump with a sterile polyethylene tube. The mini-pump was implanted subcutaneously on the back and the infusion was continued for two weeks at an infusion rate of 0.5 µl/h, unless otherwise specified. In some experiments, 8-B-cAMP was also infused after dilution to a concentration of 25 mg/pump. Three days prior to the end of the infusion, Calcein (6 mg/a rat; Sigma Co., St. Louis) was injected subcutaneously for later fluorescent microscopic examination. In each group described above, at least six rats were examined.

X-ray photographs, bone mineral assessment and histological examination

After the infusion, the femur was excised and examined by means of soft X-ray photography, bone mineral measurement and histological study to investigate the effects of PGE2 infusion. The contra-lateral femur was also examined as a control. Soft X-ray photographs were taken at 31KeV, 20 mA/s with a Senomax X-ray apparatus (CGR, France). The untreated femur was always taken in the same film as the control. Bone mineral content

(BMC) was assessed by means of dual X-ray absorptiometry (DXA; Hologic QDR-2000, Waltham MA, fine mode with a pin-hole collimator). In preliminary experiments, the ash-weights of bones were measured after heating the bones at 600 °C for 24 h in an electric oven; ash-weight was compared with the results of DXA measurements. The correlation between ash-weight and BMC assessed by DXA was good ($y=0.735x+7.969$, $r=0.978$, $n=15$, $P<0.001$). Thus, in the following experiments, BMC was assessed by DXA, which showed an excellent reproducibility (C.V. of five separate measurements =1.2%). The contra-lateral intact femur was always studied as a control, and data were expressed as a ratio of the BMC of the infused bone to that of the intact bone in the same rat.

Finally, the femur was embedded in methyl methacrylate and sagittal sections (5 µm thick) parallel to the long axis, extending from the proximal one-third to the distal one-third, were prepared. Some of these sections were stained by Goldner's method for microscopic examination and the others were used for the assessment of fluorescence labeling.

Serum biochemical analysis

At the end of the infusion, the rats were sacrificed by bleeding by cardiac puncture while anesthetized by an intra-peritoneal injection of sodium pentobarbital (25 mg/kg). Blood was saved for serum biochemical analysis. The serum calcium (Ca) concentration was measured by a colorimetric assay. Other biochemical parameters such as alkaline phosphatase and tartrate-resistant acid phosphatase were also assessed by colorimetric assays.

Statistical analysis

Statistical analysis was performed by unpaired *t*-test (Student's *t* test). A *P* value less than 0.05 was considered to be statistically significant.

Results

Intramedullary infusion

Figure 1 demonstrates the soft X-ray photographs

of the PGE₂ infused femora. There was dose-dependent sclerosis around the inserted needle tip. A dose of 10 μ g/pump PGE₂ (Fig. 1B) did not produce the sclerosis, while a dose of 100 μ g/pump PGE₂ (Fig. 1C) always produced extensive bone

sclerosis. In contrast, the infusion of dilution buffer alone and a dose of 25 mg/pump 8-B-cAMP did not produce significant sclerosis (Fig. 1A, D).

The BMCs of PGE₂-infused bones increased significantly (Fig. 2). The infusion of dilution buffer

Fig. 1. Soft X-ray photographs of the intramedullary infused femora (Treated: right, Untreated: left). The dilution buffer alone (arrow shows the site of a needle inserted) (1A), 10 μ g/pump of PGE₂ (1B) and 25 mg/pump of 8-bromo-cyclic adenosine monophosphate (1D) did not produce significant sclerosis, but 100 μ g/pump of PGE₂ produced remarkable sclerosis around the inserted needle tip (arrows) (1C). Photographs show representatives of several similar findings.

Fig. 2. BMCs of intramedullary infused femora. The infusion of dilution buffer alone, a low dose of PGE₂ (10 µg/pump) and 25 mg/pump of 8-bromocyclic adenosine monophosphate caused a slight increase in the BMCs in the femora without significant differences among these three groups. The infusion of a high dose (100 µg/pump) of PGE₂ increased BMC significantly compared with BMC in the contralateral intact femora (a: $P < 0.001$ vs. vehicle, b: $P < 0.001$ vs. 10 µg/pump of PG-E₂). Data are represented as the % increase in BMC compared to the contralateral intact bone. The data shown are the means of samples (dilution buffer $n=12$, others $n=6$) and bars represent the 1SD of the means.

alone or a low dose of PGE₂ (10 µg/pump) caused a slight increase in the BMCs of the infused femora that was probably attributable to bone formation stimulated by the surgical procedure, without a significant difference between these two groups. The infusion of a high dose (100 µg/pump) of PGE₂ caused a significant increase in BMC when compared to those of the contra-lateral intact femora ($P < 0.001$ vs. control and 10 µg/pump of PGE₂), corresponding to the findings in soft X-ray photographs. Infusion of 8-B-cAMP (25 mg/pump) did not cause an increase in BMC, corresponding to the findings in soft X-ray photographs.

The histological findings are shown in Fig. 3. The infusion of dilution buffer alone produced proliferation of fibrous tissue around the infusion site. This was accompanied with a little bone formation in its periphery. There was also minimal periosteal bone formation (Fig. 3a). Infusion of PGE₂ (100 µg/pump) produced remarkable intramedullary bone formation with extensive periosteal bone formation (Fig. 3b). In the bone marrow, small mononuclear cells replaced the fat tissue, which is

usually found in normal bone marrow, and there appeared new bone extending from the inner surface of the cortex. Osteoid was covered by cuboid shaped osteoblasts around which proliferation of spindle shaped cells was observed (Fig. 3c). In addition, many small vessels were found near the newly formed bone (Fig. 3d). The infusion of dilution buffer alone and 8-B-cAMP (Fig. 3e) did not cause the bone marrow change. Strong Calcein labeling was found all over the surface of the new bone formed in the marrow (Fig. 3f).

Infusion onto the periosteum

Figure 4 shows a soft X-ray photograph of the PGE₂-infused femora. There was a periosteal reaction on the mid-shaft of the femur (Fig. 4A), which was confirmed histologically (Fig. 4B). In addition, there appeared extensive new vessel formation sub-periosteally (Fig. 4C). In the both infusion methods, the contra-lateral femora did not show any histological changes in either the marrow or the periosteum at the concentration of PGE₂ and 8-B-cAMP used (data not shown).

The intramedullary infusion of PGE₂ and 8-B-cAMP and the infusion of PGE₂ on the periosteum caused significant changes neither in serum Ca levels, nor in other serum biochemical parameters levels such as alkaline phosphatase and tartrate-resistant acid phosphatase (data not shown).

Discussion

PGE₂ mediates the actions of several cytokines such as interleukin-1 and tumor necrosis factor and has been proven to have a potent bone resorbing activity *in vitro* [15–18]. Recent studies show, however, that PGE₂ stimulates bone formation *in vivo*. The subcutaneous infusion and oral administration of PGE₂ increased the number of osteoblasts [2, 19] and a pellet slowly releasing prostaglandin E₁ produced bone formation near the pellet [20]. The purpose of our study was to prove the effect of PGE₂ on the bone more directly and to know the mechanism of the action of PGE₂. For this purpose we performed direct intramedullary infusion of PGE₂, which should show more directly the action of PGE₂ than systemic infusion, in the adult rat femur, and examined the effects in soft

Fig. 3. Microscopic examination of intramedullary infused femora. The infusion of dilution buffer alone produced proliferation of fibrous tissue around the infusion site (arrow), accompanied by a little bone formation on its periphery and minimal periosteal bone formation (3a). The infusion of PGE₂ (100 μ g/pump) produced remarkable intramedullary bone formation with extensive periosteal bone formation (the large arrow shows the site of the needle tip, and the small arrows show bone formation in the marrow space. The star (*) shows the periosteal bone formation) (3b). Following the infusion of PGE₂ (100 μ g/pump), small mononuclear cells replaced the fat tissue and new bone was formed in the bone marrow. Osteoid was covered by cuboid shaped osteoblasts around which the proliferation of spindle shaped cells was observed (3c). In addition, many small vessels were found near the newly formed bone (3d). The infusion of 8-bromo-cyclic adenosine monophosphate did not cause the bone marrow to change (3e). Following the infusion of PGE₂ (100 μ g/pump), strong Calcein labeling was observed all over the surface of the newly formed bone (3f). (magnification; 3a, b: $\times 5$, 3c-f: $\times 50$).

Fig. 4. The soft X-ray photograph and microscopic examination of femur following the infusion of PGE2 (100 $\mu\text{g}/\text{pump}$) onto the periosteum. Extensive periosteal bone formation on the mid-shaft of the femur is seen in the soft X-ray photograph (4A). 4B shows the histological finding of the periosteal bone formation, in the rectangle in 4A. In addition, there appeared to be extensive new vessel formation sub-periosteally (4C, the magnified view of the rectangle in 4B; magnification: 4B: $\times 5$, 4C: $\times 50$).

X-ray photographs, bone mineral content measurement and histological sections.

Our study clearly demonstrated that PGE2 stimulated bone formation. By PGE2 infusion into the bone marrow, new bone was formed in the marrow around the infusion site (Fig. 3b) and BMC was increased at the infused sites. Periosteal infusion caused periosteal bone formation, while vehicle alone did not. Histologically, stimulation of bone resorption by PGE2 was not extensively observed. This might be attributable to the experimental periods in this study: activated osteoclasts may at first disappear during two weeks' infusion. In our experimental model, this was difficult to prove, because of concomitant effects of a surgical procedure early in the experiments. At least it is safe to say that chronic infusion of PGE2 results predominantly in bone formation.

We observed prominent new vessel formation around the new bone or rather new bone formation appeared around the newly formed vessels (Fig. 3d). Prostaglandin-E1 increased the intracortical remodeling space associated with increased vascularization [20]. Prostaglandin-E series are known to have actions on the vascular tissue [21–24], and thus the effects on the bone formation

might be related to its action on the vascular system.

PGE2 is a well-known activator of adenylate cyclase in many cells including osteoblastic cells [25, 26]. We therefore infused 8-B-cAMP to mimic the action of PGE2, and no significant effects of cAMP analog were observed. Recent findings show the presence of three subtypes (EP1, EP2, and EP3) of PGE2 receptors, presumed coupled to stimulation of phospholipase C, and stimulation and inhibition of adenylate cyclase [27]. In relation to the action on the bone, which subtype(s) of receptor is mainly involved is not certain. Our findings suggest that stimulation of adenylate cyclase may not be involved in the bone forming activity of PGE2. Other evidence supporting this hypothesis is that prostaglandin D2 stimulates calcification of human osteoblastic cells *in vitro* without the mediation of cAMP [28]. The mechanism of action of PGE2 on bone formation remains to be investigated.

PGE2 stimulated bone formation not only in the histological examination but also in X-ray photographs and in BMC measurement. PGE2 increased BMC in the infused bone. Since new bone is formed in the bone marrow without prior bone trabeculae, the injection of PGE2 into the bone mar-

row would have a therapeutic potential in patients with senile osteoporosis, in which trabecular loss or discontinuity is thought to hamper the therapeutic responses due to various drugs. Local administration of PGE₂ in our experiments did not cause side effects in rats.

In conclusion, we have shown the direct stimulation of bone formation of PGE₂.

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