FEBS Letters 581 (2007) 565–571

Prostaglandin E receptor EP4 antagonist suppresses osteolysis due to bone metastasis of mouse malignant melanoma cells

Morichika Takita[a], Masaki Inada[a], Takayuki Maruyama[b], Chisato Miyaura[a,*]

[a] *Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2-24-16 Nakamachi, Koganei, Tokyo 184-8588, Japan*

[b] *Discovery Research Laboratory I, Minase Research Institute, Ono Pharmaceutical Co. Ltd., Osaka, Japan*

Received 30 October 2006; revised 30 December 2006; accepted 6 January 2007

Available online 16 January 2007

Edited by Richard Marais

Abstract **We examined the effects of prostaglandin E (PGE) receptor subtype EP4 antagonist on bone metastasis of cancer to clarify PGE's role in bone metastasis. Metastatic regions were detected in femurs accompanying severe bone loss in mice injected with B16 malignant melanoma cells. Administration of EP4 antagonist restored the bone loss induced by B16 melanoma. Adding B16 cells induced osteoclast formation in the coculture of bone marrow cells and osteoblasts without any exogenous bone-resorbing factor, and EP4 antagonist completely suppressed the osteoclast formation induced by B16 cells. Therefore, EP4 antagonist is a possible candidate for the therapy of bone metastasis of cancer.**

Keywords: Prostaglandin E; EP4 receptor; Bone metastasis of cancer; Bone resorption; Malignant melanoma

1. Introduction

Bone metastasis of cancer is accompanied by severe bone destruction with increased bone resorption. Some cancer cells are known to release soluble bone-resorbing factors such as parathyroid hormone-related protein (PTHrP), which may be one of the candidates for regulators in tumor-induced osteolysis [1,2]. On the other hand, cell-to-cell interaction between cancer and host cells is thought to be involved in the mechanism of bone resorption in the region of cancer metastasis. We have reported that bone tissue with metastasis of breast cancer highly expressed receptor activator of NFκB ligand (RANKL), a key molecule for osteoclast differentiation, and that the expression of RANKL in osteoblasts was enhanced by contact with the cancer cells in vitro [3], suggesting that the regulation of host cells is a candidate therapeutic approach for bone metastasis of cancer.

*Corresponding author. Fax: +81 42 388 7390.
E-mail address: miyaura@cc.tuat.ac.jp (C. Miyaura).

Abbreviations: PGE, prostaglandin E; RANKL, receptor activator of NFκB ligand; TRAP, tartrate-resistant acid phosphatase; COX, cyclo-oxygenase; PTHrP, parathyroid hormone-related protein; NSAID, non-steroidal anti-inflammatory drugs; BMD, bone mineral density; BV/TV, bone volume/tissue volume; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; ES/BS, erosion surface/bone surface; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Prostaglandin E2 (PGE2) is produced in bone mainly by osteoblasts and stimulates bone resorption. There are four subtypes of PGE receptors, designated EP1, EP2, EP3, and EP4, that are encoded by different genes and expressed differently in each tissue [4–7]. The intracellular signaling differs among the receptor subtypes; EP1 is coupled to calcium mobilization, EP3 inhibits adenylate cyclase, whereas both EP2 and EP4 stimulate adenylate cyclase in various types of cells. Using knockout mice of respective EP and specific EP agonists, we reported that PGE2 stimulates bone resorption mainly by EP4 [8,9]. EP4 agonist greatly stimulated the expression of RANKL in osteoblasts and induced osteoclast formation in mouse bone marrow cultures [8]. Therefore, PGE2 may stimulate bone resorption by the RANKL-dependent mechanism via EP4 receptors expressed in osteoblasts.

Previous studies suggest a possible correlation between cancer growth and prostaglandins (PGs). Null mutation of the cyclo-oxygenase (COX)-1, COX-2, and EP2 genes showed reduced intestinal polyp formation in Min mice with a mutation in the *Apc* gene [10,11]. Non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to reduce the risk of breast cancer and colon carcinogenesis [12,13]. Some malignant tumor cells highly express COX-2 in vivo, and COX-induced PGE2 production enhances the tumorigenesis of cancer [14]. Recently, Ma et al. [15] reported that EP4 antagonist inhibits the PGE2-induced chemotactic response and lung metastasis of breast cancer cells. Previous studies have shown that the expression of COX-2 was elevated in host stromal cells and osteoblasts in the region of bone metastasis of cancer [16]. Therefore, suppression of the PGE signal in host cells may be a possible way to improve severe osteolysis due to the bone metastasis of cancer.

In the present study, we examined the effects of EP4 antagonist on bone destruction due to the metastasis of malignant melanoma, and show that blockage of the EP4 signal is a new therapeutic approach for bone destruction due to cancer metastasis.

2. Materials and methods

2.1. Intracardiac injection of B16 cells in C57BL/6 mice

B16 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum (FCS) at 37 °C under 5% CO2 in air. B16 cells (2×10^5 cells) were suspended in 0.1 mL PBS and injected into the left heart ventricle of 6-week-old male C57BL/6 mice (Shizuoka, Japan) under anesthesia with pentobarbital. Animals were kept in our clean animal facilities for 12 days. EP4 antagonist, AE3-208, was prepared in Ono pharmaceutical Co. Ltd. The K_i values

0014-5793/$32.00
doi:10.1016/j.febslet.2007.01.005

of AE3-208 obtained by competition-binding isotherms to displace the radio-labeled ligand binding to the respective prostanoid receptor are 1.3, 30, 790, and 2400 nM for EP4, EP3, FP, and TP, respectively, and more than 10 000 nM for the other prostanoid receptors [17]. EP4 antagonist (10 mg/kg of body weight/day) was administered by oral gavage to mice from days 0 to 11, and the femurs were collected from the mice on day 12. The dose usage of EP4 antagonist was referred previous study for experimental mouse colitis model [17]. As a control group, mice were administered distilled water only. All experimental procedures were performed in accordance with institutional guideline for animal research, and approved by the Committee for Animal Research in Tokyo University of Agriculture and Technology.

2.2. Measurement of bone mineral density

The bone mineral density (BMD) of the femurs was measured by dual X-ray absorptiometry (model DCS-600R; Aloka), as previously reported [18]. The bone mineral content of the femurs was closely correlated with the ash weight [18]. The BMD was calculated by dividing the bone mineral content of the measured area by the area.

2.3. Measurement of PGE2 content in bone marrow supernatant

Six-week-old C57BL/6 mice were injected with B16 cells and the femurs and tibiae were collected 12 days after the injection. To obtain the bone marrow supernatant, bone marrow cells and trabecular bone fragments were collected with 1 mL of PBS from the femurs and tibiae, as previously reported [19]. After centrifugation to remove the cells and bone fragments, the supernatant was collected for the measurement of PGE2. The concentration of PGE2 in the bone marrow supernatant was determined using an enzyme immunoassay (EIA; Amersham Biosciences) with the standard curve in the range of 50–6400 pg/mL.

2.4. Histological analysis of the femoral trabecular bone

The distal metaphysis of the femur was fixed with 70% ethanol and embedded in glycol methacrylate, and undecalcified 3-μm sections were prepared and stained for hematoxylin–eosin (HE), as reported previously [18]. The trabecular bone volume density (bone volume/tissue volume [BV/TV]), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), and erosion surface/bone surface (ES/BS) were determined in the trabecular bone at the secondary spongiosa of the distal metaphysis in femurs [18].

2.5. RT-PCR analysis

Total RNA was extracted from the femur and cocultured mouse osteoblasts and bone marrow cells, using the acid guanidium–phenol–chloroform method [20]. cDNA was synthesized from 5 μg of total RNA by reverse transcriptase (Superscript II Preamplification System, Invitrogen, Carlsbad, CA) and amplified via PCR. The primers in PCR for the mouse RANKL, COX-1, COX-2, EP4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were used as reported previously [20]. The PCR product was run on a 1.5% agarose gel and stained with ethidium bromide.

2.6. Osteoclast formation in coculture of mouse bone marrow cells and osteoblasts

Primary osteoblastic cells were isolated from 2-day-old mouse calvariae, as described previously [18]. Bone marrow cells (3×10^6 cells) were isolated from 6-weeks-old mice and cocultured with the primary osteoblastic cells (1×10^4 cells) in 1 mL of αMEM containing 10% FCS with a physiological concentration (10 pM) of 1α,25-dihydroxyvitamin D3. To examine the effects of B16 cells on osteoclast formation, B16 cells were fixed with 4% paraformaldehyde, washed three times with PBS, and added to the cocultures. After being cultured for 7 days, the cells were stained for tartrate-resistant acid phosphatase (TRAP), and TRAP-positive multinucleated cells were counted as osteoclasts. To extract total RNA for RT-PCR analysis, the cells adhering to the well surface were lysed on day 4. Data are expressed as the means ± S.E.M. The significance of differences was analyzed using Student's *t*-test.

2.7. Colony formation of B16 cells

B16 cells (3×10^2 cells) were cultured in 2 mL of DMEM containing 10% FCS on six-well plates. The cultures were maintained by replacing the old medium to fresh medium for every 3 days. After being cultured for 7 days, the cells adhering to the well surface were stained for crystal violet to visualize colonies. The number of B16 cell colonies was counted using microscopy.

3. Results

3.1. Bone loss and increased PGE2 production in bone with metastasis of malignant melanoma cells

We first established the experimental model of bone metastasis of cancer cells using mouse B16 melanoma, and measured the bone density of femurs. B16 cells were injected into the left heart ventricle of C57BL/6 mice and the femurs were collected from the mice to measure BMD of the femurs. Since B16 cells actively produced melanin, the metastasis region could be detected as black in the femurs on day 12 after the injection of B16 cells (Fig. 1A). The BMD was significantly reduced in mice with bone metastasis of B16 compared with control mice (Fig. 1D). In RT-PCR using total RNA extracted from femur, the mRNA expression of RANKL and COX-2 was elevated in the femur with metastasis of B16 cells compared with control (Fig. 1B). To examine the possible involvement of PGE2 production in bone loss due to metastasis, we collected bone marrow supernatant from the femur and tibia, and measured the PGE2 level. The level of PGE2 in bone

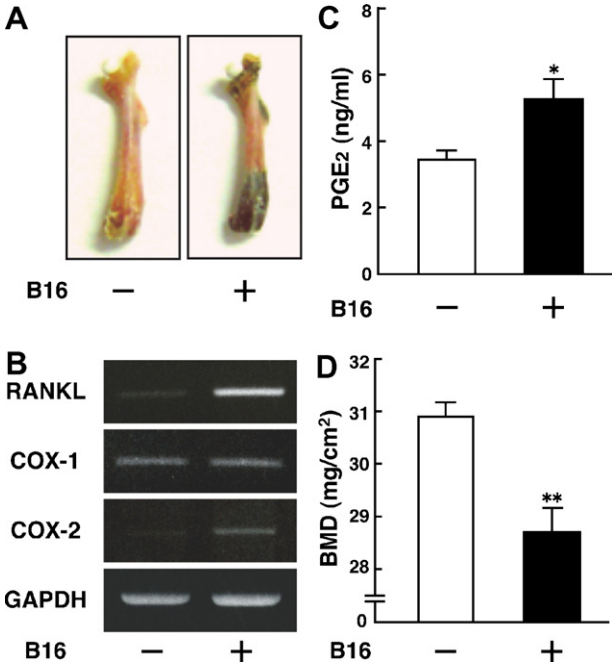

Fig. 1. Intracardiac injection of B16 cells into C57BL/6 mice causes severe osteolysis due to bone metastasis. (A) C57BL/6 mice were injected with or without B16 cells and the femurs were collected from mice on 12 days after injection. Representative pictures of the femur are shown. (B) The expression of RANKL, COX-1, and COX-2 mRNA was analyzed by RT-PCR using total RNA collected from the femur with or without B16 metastasis. (C) The bone marrow fluid was prepared using 1 mL of PBS, and the concentration of PGE2 in the bone marrow fluid was determined using an EIA. Significantly different from control mice without B16 cell injection, $^{*}P < 0.05$. Data are expressed as the means ± S.E.M. of 6–7 mice. (D) The BMD was measured at the total area of the femur. Significantly different from control mice without B16 cell injection, $^{**}P < 0.01$. Data are expressed as the means ± S.E.M. of 6–7 mice.