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

Moricihika Takita\textsuperscript{a}, Masaki Inada\textsuperscript{a}, Takayuki Maruyama\textsuperscript{b}, Chisato Miyaura\textsuperscript{a,\ast}

\textsuperscript{a} Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2-24-16 Nakamachi, Koganei, Tokyo 184-8588, Japan

\textsuperscript{b} Discovery Research Laboratory I, Minase Research Institute, Ono Pharmaceutical Co. Ltd., Osaka, Japan

Abstract We examined the effects of prostaglandin E (PGE) receptor subtype EP4 antagonist on bone metastasis of cancer to clarify PGE\textsuperscript{\ast} 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.

\copyright 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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 NF\textsuperscript{\kappa}B 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.

Prostaglandin E2 (PGE\textsubscript{2}) 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 PGE\textsubscript{2} 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, PGE\textsubscript{2} 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 \textit{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 PGE\textsubscript{2} production enhances the tumorigenesis of cancer [14]. Recently, Ma et al. [15] reported that EP4 antagonist inhibits the PGE\textsubscript{2}-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 blockade 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\% CO\textsubscript{2} in air. B16 cells (2 × 10\textsuperscript{4} 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.

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

Available online 16 January 2007

Edited by Richard Marais

0014-5793/32.00 © 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2007.01.005

Abbreviations: PGE, prostaglandin E; RANKL, receptor activator of NF\textsuperscript{\kappa}B ligand; TRAP, tartrate-resistant acid phosphatase; COX, cyclo-oxygenase; PTHrP, parathyroid hormone-related protein; NSAID, non-steroidal anti-inflammatory drugs; BMD, bone mineral density; COX, cyclo-oxygenase (COX)-1, COX-2, and EP2 genes showed reduced intestinal polyp formation in Min mice with a mutation in the \textit{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 PGE\textsubscript{2} production enhances the tumorigenesis of cancer [14]. Recently, Ma et al. [15] reported that EP4 antagonist inhibits the PGE\textsubscript{2}-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 blockade 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\% CO\textsubscript{2} in air. B16 cells (2 × 10\textsuperscript{4} 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 \textit{Ki} values

0014-5793/32.00 © 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
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 10000 nM for the other prostanoid receptors [17]. EP4 antagonist (10 mg/kg of body weight/day) was administered by oral gavage from days 0 to 11, and the femurs were collected from the mice on day 12. The dose usage of EP4 antagonist was referred from 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 guidelines for animal research, and approved by the Committee for Animal Research in Tokyo University of Agriculture and Technology.

2.5. RT-PCR analysis
physis in femurs [18]. in the trabecular bone at the secondary spongiosa of the distal met.

2.4. Histological analysis of the femoral trabecular bone
The distal metaplasia 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]) of the trabecular bone 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.

2.2. 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.

2.1. Measurement of bone mineral content
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.

2.6. 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-week-old mice and cocultured with the primary osteoblastic cells (1 × 10^6 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 × 10^5 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 induced 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.
Fig. 2. Oral administration of EP4 antagonist suppresses osteolysis due to bone metastasis of B16 cells. C57BL/6 mice were injected with or without B16 cells and then EP4 antagonist was administered by oral gavage to mice injected with or without B16 cells for 11 days. Control mice injected with or without B16 cells were administered with distilled water. The femurs were collected from mice 12 days after injection. The BMD was measured at the total area of the femur. Significantly different, *P < 0.05. Data are expressed as the means ± S.E.M. of 6–8 mice.

marrow supernatant significantly increased in mice with bone metastasis (Fig. 1C). These results suggest that PGE2, a potent bone-resorbing factor, produced by bone with metastasis is involved in the mechanism of bone loss due to the metastasis of B16 cells.

3.2. Effects of EP4 antagonist on BMD of femur with metastasis of B16 cells

In the experimental model of bone metastasis using B16 melanoma cells, we examined the effects of EP4 antagonist on femoral BMD in mice with metastasis. When EP4 antagonist was orally administered to mice injected with B16 cells, decreased BMD was significantly restored (Fig. 2). Administration of EP4 antagonist into mice without injection of B16 cells had no effect on femoral BMD.

3.3. Histological analysis of bone loss due to metastasis of B16 cells

In the section stained for HE, metastatic tumor cells filled the bone marrow cavity and trabecular bone area in the distal femoral metaphysis of mice injected with B16 cells (Fig. 3A). Trabecular bone was surrounded by tumor cells, and was markedly destroyed in these mice (Fig. 3B). Sections prepared from mice treated with EP4 antagonist showed a reduced area of tumor metastasis, and trabecular bone loss was attenuated (Fig. 3A and B). Next, we performed histological analysis of trabecular bone using the section of the distal femoral metaphysis. In mice injected with B16 cells, BV/TV and Tb.Th were significantly reduced, whereas Tb.Sp increased compared with control mice without the injection of B16 cells (Fig. 3C–E). The increase in Tb.Sp indicates that osteoclastic bone resorption was stimulated, resulting in enhanced intertrabecular space. In fact, ES/BS significantly increased in the trabecular bone obtained from mice with bone metastasis compared with control mice without metastasis (Fig. 3F). By the administration of EP4 antagonist to mice injected with B16 cells, the reduced BV/TV was significantly elevated, and the changes of Tb.Th, Tb.Sp, and ES/BS were restored. Therefore, the metastasis of B16 induced the loss of trabecular bone by increased bone resorption, and EP4 antagonist attenuated bone destruction due to cancer metastasis.

3.4. Osteoclast formation induced by B16 melanoma cells in vitro

To examine the action mechanism of EP4 antagonist in bone resorption due to cancer metastasis, we performed the cocultures of bone marrow cells and osteoblasts in the presence or absence of B16 cells fixed with paraformaldehyde. By the use of fixed-B16 cells instead of live B16 cells, we excluded the possibility of the influence of soluble factor(s) produced by B16 cells, prevented the growth of B16, and specifically focused on the cell-to-cell interaction between cancer cells and host osteoblasts in the cocultures. B16 cells markedly induced osteoclast formation in the cocultures, and adding EP4 antagonist completely suppressed osteoclast formation (Fig. 4A). COX-2 inhibitor, NS398, and indomethacin also suppressed osteoclast formation induced by B16 cells. The level of PGE2 was markedly elevated by the cocultures with B16, and suppressed by NS398 and indomethacin, but not by EP4 antagonist (Fig. 4B). The expression of RANKL mRNA was enhanced by coculture with B16, and the expression was suppressed by EP4 antagonist (Fig. 4C). NS398 and indomethacin also suppressed the expression of RANKL mRNA in the cocultures. PGE2 is produced by osteoblasts after cell-to-cell interaction with B16 cells, and plays a critical role in tumor-induced osteoclastogenesis, and that EP4 antagonist acts on osteoblasts to block the binding of PGE2 to EP4 receptors expressed in osteoblasts following the suppression of osteoclast formation.

3.5. Effects of PGE2 and EP4 antagonist on cell growth of B16 melanoma in vitro

To examine the effects of PGE2 and EP4 antagonist on the proliferation of B16 cells, we added PGE2 and EP4 antagonist to the cultures of B16 cells, and counted the number of colonies on day 7. Neither PGE2 nor EP4 antagonist influenced the number of B16 colonies (Fig. 5A). We further examined whether B16 cells expressed COX-2 and EP4. In RT-PCR, the expression of COX-1, COX-2, and EP4 mRNAs was only detected in mouse osteoblasts but not in B16 cells (Fig. 5B). In addition, PGE2 could not be detected in the conditioned medium of B16 cell cultures (data not shown). Therefore, it is unlikely that the growth of B16 is directly regulated by PGE2.

4. Discussion

In the present study, the administration of EP4 antagonist clearly suppressed osteolysis in the region of bone metastasis in mice injected with malignant melanoma B16 cells (Figs. 2 and 3). We suggest that local production of PGE2 in bone and bone marrow may contribute to the loss of trabecular bone in femurs with metastasis. To define whether PGE2 acts on B16 cells to regulate cell growth, we examined the effects of PGE2 and/or EP4 antagonist on colony formation of B16 cells in vitro. Neither PGE2 nor EP4 antagonist affected the cell growth of B16 cells (Fig. 5A). Recently, Xia et al. reported that...
PGE2 did not affect the proliferation of B16 cells in MTT assay [21]. In addition, B16 cells did not express COX-2 and EP4 mRNAs (Fig. 5B). These results suggest that PGE2 produced by host cells act on osteoblasts in bone tissue to stimulate bone...
resorption at the site of metastasis, and that EP4 antagonist blocks the binding of PGE2 to osteoblasts, resulting in the suppression of osteoclastic bone resorption.

Previous studies have shown that PTHrP produced by cancer cells is implicated in osteolysis due to cancer metastasis [1,2]. Then, we examined the expression of PTHrP in B16 cells, and found no expression of PTHrP mRNA in B16 cells (data not shown). Perez et al. also reported the lack of PTHrP production by B16 cells [22]. Therefore, it is unlikely that PTHrP is a regulator for bone metastasis of cancer in our experimental model.

It is well known that PGE2 acts on osteoblasts and induces the expression of RANKL to elicit osteoclastogenesis. We have reported that PGE2 could not induce bone resorption in organ cultures of mouse calvaria collected from EP4-null mice, and that EP4 agonist stimulates RANKL expression in osteoblasts and osteoclast formation in bone marrow cultures [8,9]. Therefore, PGE2 may stimulate bone resorption by the RANKL-dependent pathway via the EP4 receptor subtype expressed in osteoblasts. Ono et al. [16] have shown that bone marrow stromal cells adjacent to tumor cells express COX-2.
protein at the site of bone metastasis. We have also shown that cell-to-cell interactions between breast cancer cells and osteoblasts induced the expression of COX-2 in osteoblasts [23]. In the present study, adding B16 cells could induce osteoclast formation in the coculture of bone marrow cells and osteoblasts (Fig. 4). In the cocultures, B16 cells were fixed with paraformaldehyde to eliminate the influence of soluble factor(s) produced by B16. Then, we focused on the cell-to-cell interaction between B16 and osteoblasts, that was probably mediated by cell surface molecule(s) on their membrane. In this study, we propose a model of the mechanism by which cell-to-cell contact between cancer cells and host osteoblasts induces PGE2-mediated osteoclast formation in bone with osteolytic metastasis. The contact of cancer cells with osteoblasts induces COX-2 to produce PGE2, and PGE2 binds to the EP4 receptor expressed in osteoblasts, resulting in the expression of RANKL in osteoblasts to induce osteoclasts formation from monocyte-macrophages. The PGE2-EP4 signaling may be critical for cancer-induced progression of osteoclastic bone resorption in the bone metastasis of cancer. Further studies are necessary to define a cell surface molecule(s) involved in the interaction between B16 and osteoblasts, and the mechanism of signal transduction after the cell-to-cell contact to produce PGE2 by osteoblasts.

Epidemiological studies and clinical observations suggest that non-steroidal anti-inflammatory drugs (NSAIDs) may reduce the risk of certain types of human cancer [12,13]. Recently, Gupta et al. [24] have reported that the administration of celecoxib, a selective COX-2 inhibitor, suppressed prostate carcinogenesis in transgenic adenocarcinoma of the mouse prostate model. COX-2 inhibitors have also been reported to act as chemopreventive agents against colon carcinogenesis [13]. NSAIDs are also known to reduce the risk of breast cancer, and elevated COX-2 expression was associated with tumor size in patients with breast cancer [12]. Amano et al. [25] have reported that implanted tumor growth associated with angio genesis was markedly suppressed in EP3-null mice, and that the administration of COX-2 inhibitor reduced tumor growth in wild-type mice. In conclusion, the present study clearly showed that the administration of EP4 antagonist suppressed the area of metastasis of B16 in bone and bone loss in the metastatic region in vivo. Therefore, PGE2 produced by host osteoblasts may be involved in osteolysis due to tumor cells. The present study supports the development of clinical trials to determine whether EP4 antagonist can be useful as a therapeutic agent in the management of cancer accompanying bone metastasis.

References


