Abrogation of prostaglandin E-EP4 signaling in osteoblasts prevents the bone destruction induced by human prostate cancer metastases

Kenta Watanabe[1], Tsukasa Tominari[2, 3], Michiko Hirata[2], Chiho Matsumoto[2], Takayuki Maruyama[4], Gillian Murphy[3, 5], Hideaki Nagase[3, 6], Chisato Miyaura[1, 2, 3] and Masaki Inada[1, 2, 3]

[1]Cooperative Major of Advanced Health Science, [2]Department of Biotechnology and Life Science, [3]Institute of Global Innovation Research, Tokyo University of Agriculture and Technology, 2-24-16 Nakacho, Koganei, Tokyo 184-8588, Japan, [4]Minase Research Institutes, Ono Pharmaceutical Co. Ltd, Osaka 618-8585, Japan, [5]Department of Oncology, University of Cambridge, Cancer Research UK, Cambridge Institute, Li Ka Shing Centre, Cambridge, CB2 0RE, UK, and [6]Kennedy Institute of Rheumatology, Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Oxford, OX3 7FY, UK

Address correspondence and reprint requests to: Masaki Inada, Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2-24-16 Nakacho, Koganei, Tokyo 184-8588, Japan. Phone No.:81-42-388-7402, FAX No.: 81-42-388-7390, E-mail address: m-inada@cc.tuat.ac.jp

Abstract

The metastasis of tumors to bone is known to be promoted by prostaglandin E2 (PGE2) produced by the tumor host stromal tissue. Although bone metastases frequently occur in prostate cancer patients, the significance of PGE2 in stromal responses to the tumor is not known. In this study, we report that PGE2 and its receptor EP4 play a pivotal role in bone destruction and metastasis in an experimental metastasis model of prostate cancer in nude mice. Using human prostate cancer PC-3 cells that are stably transfected with luciferase, we showed that the development of bone metastasis was accompanied by increased osteoclastic bone resorption in the bone metastasis microenvironment, and could be abrogated by an EP4 receptor antagonist. The growth of PC-3 cells in vitro was not influenced by PGE2 or by the EP4 receptor. However, cell-cell interactions between fixed PC-3 cells and host osteoblasts induced PGE2 production and RANKL expression in the osteoblasts. Addition of an EP4 antagonist suppressed both PGE2 and RANKL expression induced by the PC3-osteoblast interaction, which would have consequent effects on osteoclast activation and osteolysis. These results indicate that the blockage of PGE2-EP4 signaling prevents the bone destruction required for prostate cancer metastases, and that this is, in part due to the abrogation of bone cell responses. The study provides further evidence that an EP4 antagonist is a candidate for the treatment of prostate cancer in the blockade of bone metastasis.

Keywords: Prostate cancer, Bone metastasis, Prostaglandin E, Bone resorption, EP4 antagonist

1. Introduction

Prostate cancer is a common disease of older men, and its incidence is the second highest worldwide [1]. Bone metastasis is a frequent occurrence in prostate cancer patients, and cancer-induced bone responses favor the survival and growth of cancer cells in the new environment [2]. Patients with advanced prostate cancer show sclerotic bone metastases, which cause chronic pain and pathologic fractures [3]. However, the invasion of prostate cancer cells into bone tissues firstly induces bone destruction by increased osteoclast-mediated bone resorption [4]. Hence, bisphosphonates such as zoledronic acid, have been used to prevent the development of metastatic bone lesions in patients with prostate cancer [5], but the outcome of such treatment is still largely unsatisfactory.

Prostaglandin E2 (PGE2) is an inflammatory mediator produced by various cell types and is known to have effects on aspects of tumorigenesis, survival and invasiveness [6]. PGE production is regulated by three metabolic steps; the release of arachidonic acid from membrane phospholipids by phospholipase A2 (PLA2), the conversion of arachidonic acid to PGH2 by cyclooxygenases (COX), and the synthesis of PGE2 by PGE synthases (PGES). Increased expression of COX-2 and membrane-bound PGES-1 (mPGES-1) have been implicated in various inflammatory diseases [7]. We have reported that the bone resorption associated with inflammation was attenuated in cPLA2-deficient mice and in mPGES-1-deficient mice due to the lack of PGE production by osteoblasts in bone tissues [8, 9]. Recently, we have reported the role of PGE2 derived from normal host cells on the growth and metastasis of malignant melanoma using mPGES-1-deficient mice [10].

It is well known that the receptor activator of NF-κB ligand (RANKL) is a pivotal factor in osteoclast differentiation and bone resorption, and that osteoblasts express RANKL in response to bone-resorbing factors and interact with osteoclast precursors expressing the receptor activator of NF-κB (RANK) to induce differentiation into

osteoclasts [11]. Previous studies have shown that an anti-RANKL antibody suppressed the bone destruction with the bone metastasis of cancer, and the occurrence of skeletal-related events in both animals and humans [12, 13]. We have reported that human breast cancer cells induced RANKL expression in osteoblasts and stimulated osteoclastogenesis [14]. Therefore, it is possible that the bone metastasis of prostate cancer is closely related to the RANK-RANKL axis in bone tissues.

PGE2 signals through a family of G-protein-coupled receptor subtypes identified as EP1, EP2, EP3 and EP4 in individual target cells [15]. Using EP4-deficient mice and specific agonists for the respective EPs, we have previously reported that PGE2 binds to the EP4 receptor of osteoblasts to initiate RANKL-dependent osteoclast formation [16, 17]. Using selective agonists for the respective EPs, we showed that an EP4 agonist clearly induced osteoclast formation *in vitro*. We have also reported that the growth of B16 melanoma is attenuated in EP4-deficient mice [10].

In the present study, we examined the roles of PGE-EP4 signaling in bone metastasis and bone destruction using an experimental bone metastasis model of prostate cancer in nude mice. We established the luminescence detection system to detect metastasis of human prostate cancer, PC-3 cells, and found that oral administration of an EP4 antagonist blocked the frequency of bone metastasis and bone loss in an experimental metastasis model. In vitro studies confirmed that the PGE-EP4 axis is essential for host osteoblast/osteoclast cell responses to tumor cell stimuli.

2. **Materials and Methods**

2.1. Animals and reagents.

Nude mice, BALB/c nu/nu, were obtained from Japan SLC Inc. (Shizuoka, Japan). All procedures were performed in accordance with institutional guidelines for animal research at the Tokyo University of Agriculture and Technology. PC-3, a human prostate cancer cell line, was obtained from American Type Culture Collection (ATCC), and we isolated a clone which consistently underwent a high frequency of bone metastasis. PC-3 cells were cultured in RPMI1640 medium with 10% fetal bovine serum (FBS) at 37 ^{o}C under 5% CO2 in air.

2.2 Metastasis model of PC-3 cells in nude mouse

PC-3 cells were transfected with the luciferase cDNA using plasmid pGL4.51 by Neon Transfection System (Thermo Fisher Scientific), and the cells expressing luciferase were selected by G418, and clones highly expressing luciferase were isolated in vitro.

PC-3 cells (2×10^6) were suspended in 0.1 ml of PBS and injected into the left heart ventricle of 6-week-old male nude mice. To detect the metastases, mice were monitored by the luminescence signal 5 min after the administration of luciferin intraperitoneally using an In Vivo Imaging System FX (Kodak). The bones of mice were also detected by soft X-ray in the In Vivo Imager system with the luminescence signals. The EP4 antagonist (AE3-208) was provided by Ono Pharmaceutical Co., Ltd., and was administered by oral gavage to mice (10 mg/kg of body weight/day). As a control group, mice were administered with distilled water.

2.3 Micro CT analysis

CT scanning of the femurs and tibiae was performed using a microfocus X-ray CT system (inspeXio SMX-90T; Shimadzu). Three-dimensional microstructural image data

were reconstructed, and structural indices (bone volume/tissue volume [BV/TV], trabecular thickness [Tb.Th], bone surface/bone volume [BS/BV] and trabecular separation [Tb.Sp]) were calculated using the TRI/3D-BON software program (Ratoc System Engineering Co., LTD).

2.4 Histological analysis

Tibiae were collected from nude mice and decalcified using 18% EDTA for 10 days, and embedded in paraffin, and the sections were prepared and stained by HE and TRAP to detect osteoclasts.

2.5 Culture of primary mouse osteoblastic cells and PC-3 cells

Primary osteoblastic cells were isolated from 2-day-old mouse calvariae, as described previously [9]. In some co-culture experiments, PC-3 cells or osteoblasts were fixed with 4% paraformaldehyde and washed three times with PBS. Osteoblasts were cultured for 24 h on the layer of fixed PC-3 cells. On the other hand, PC-3 cells were co-cultured with fixed osteoblasts. Using the conditioned medium of these cultures, the concentration of PGE2 was determined using an enzyme immunoassay (EIA; GE Healthcare).

2.6 Colony formation of PC-3 cells

PC-3 cells (200 cells) were plated in 12 well plates and cultured for 9 days with or without PGE2 and EP4 antagonist. After culture, the cells were stained with crystal violet and the number of colonies were counted.

2.7 RT-PCR analysis

Total RNA was extracted from osteoblasts cultured on a layer of fixed PC-3 cells or from PC-3 cells, and cDNA was synthesized from total RNA and amplified by

PCR [9]. The PCR primers for mouse RANKL were used as reported [14], and the primers for the human respective EPs (EP1, EP2, EP3 and EP4), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were used as followed: human EP1: 5'-TATCTGCTGGAGTCCCTTGC-3' (forward) and 5'-CCTCCCAA GTGCTCTTGTTT-3' (reverse), human EP2: 5'-GTTCCACGTGTTGGTGACAG-3' (forward) and 5'-ACTCGGCGCTGGTAGAAGTA-3' (reverse), human EP3: 5'-GTCCTCTACCTGTCCCACCA-3' (forward) and 5'-TGTGGCTTGAGTACCAGTGC-3' (reverse), human EP4: 5'-TCGTGGTGCTCTGTAAATCG-3' (forward) and 5'-CTCATCGCACAGATGATGCT-3' (reverse), human GAPDH: 5'-CATGGAGAAGGCTGGGGCTC-3' (forward) and 5'-AGGCAGG GATGATGTTCTGG-3' (reverse). The PCR product was run on a 1.5% agarose gel and stained with ethidium bromide. The band intensity was measured by densitometric analysis using Image J.

2.8 Statistical analysis

The data are expressed as the mean $\pm$ SEM. The significance of differences was analyzed using the Tukey test.

3. Results

3.1. Oral administration of EP4 antagonist prevent bone metastasis of PC-3

PGE2 is known to act on individual target cells through a family of receptor subtypes identified as EP1, EP2, EP3 and EP4. We have reported that the EP4 receptor mediates PGE action in bone tissues especially osteoclastic bone resorption [16, 17]. Using the specific antagonist for EP4, AE3-208, we evaluated the role of PGE-EP4 signaling in the incidence of metastasis to various tissues including bone in an experimental prostate cancer model. Nude mice were injected with PC-3-Luc cells, with and without administration of the EP4 antagonist for 26 days and metastasis was monitored by the detection of luminescence signals using an in vivo imaging system. The metastasis of PC-3-Luc was detected in some tissues, and bone metastasis was clearly detected in the region of joints between the femur and tibia by the merging of soft-X ray and luminescence signals (Fig. 1A). The EP4 antagonist markedly suppressed the metastasis of PC-3-Luc in various tissues compared with the vehicle, and the luminescence intensity elevated by the metastasis of PC-3-Luc was clearly suppressed by the treatment of EP4 antagonist (Fig. 1A).

Histological analyses of the long bones of mice showed that the trabecular and cortical bone in the proximal tibia was considerably reduced where PC-3 metastases were present, with an increased number of TRAP-positive osteoclasts. In the presence of the EP4 antagonist, AE3-208, the osteoclastic bone loss in trabecular bone was clearly restored to normal, and only a small tumor was detected in the trabecular bone area (Fig. 1B). By 3D micro-CT in the proximal region of the tibiae, severe bone destruction was detected in the tibiae by bone metastasis of PC-3, and the administration of AE3-208 clearly restored the bone loss (Fig. 1B).

3.2. EP4 antagonist restores the bone destruction due to the metastasis of PC-3

In the experimental metastasis of PC-3-Luc in nude mice, the bone architecture of the femurs was compared with controls. Using micro CT scans, to analyze the three-dimensional microstructure, we found that the trabecular bone in the femoral distal metaphysis was reduced by the metastasis of PC-3 cells in mice. However, the microstructure of the trabecular bone was restored to normal by the administration of the EP4 antagonist (Fig. 2A). Using micro CT images, we measured the structural indices of trabecular bone, BV/TV, BS/TS, Tb.Th and Tb.Sp. In control mice, the BV/TV and Tb.Th were markedly reduced, and the BS/TS and Tb.Sp were elevated by the metastasis of PC-3 prostate cancer, but these changes did not occur in mice administered with AE3-208 (Fig. 2B). These data indicate that the bone metastasis of PC-3 cells induced osteolytic bone resorption in which PGE-EP4 signaling is essential for the tumor mediated bone destruction in the tumor microenvironment.

3.3. Cell contact with PC-3 cells induces PGE production and RANKL expression in osteoblasts

In the tumor microenvironment, interactions between host cells and tumor cells is critical to understand the phenomenon of metastasis. To assess the role of cell-to-cell interactions between host cells and prostate cancer cells, osteoblasts were co-cultured with fixed PC-3 cells *in vitro*. The level of PGE2 in the conditioned medium of the co-cultures was elevated by contact with fixed PC-3 cells (Fig. 3A). The PGE2 production in osteoblasts co-cultured with fixed PC-3 cells was not influenced by adding EP4 antagonist (data not shown). PC-3 cells produce a small amount of PGE2, and the PGE production was not influence by the contact with fixed osteoblasts (Fig. 3A).

To detect the possible action of PGE and EP4 antagonist in PC-3 cells, we examined the expression of EPs in PC-3 cells, and the effects of PGE and EP4 antagonist on the growth of PC-3 cells *in vitro*. In RT-PCR, PC-3 cells express both EP2 and EP4 mRNA (Fig. 3B). The colony formation of PC-3 cells was detected in the cultures, and

adding PGE2 or EP4 antagonist did not influence the number of colony of PC-3 cells (Fig. 3C).

The expression of RANKL, a key molecule for osteoclasts differentiation, may be essential for bone loss induced by prostate cancer PC-3. In RT-PCR, the expression of RANKL mRNA in osteoblasts was clearly enhanced by the contact with fixed PC-3 cells for 24 h in the co-cultures (Fig. 4A). The RANKL expression in osteoblasts elicited by fixed PC-3 was suppressed by adding the EP4 antagonist (Fig. 4A).

4. Discussion

PGE2 is an inflammatory mediator, but is also known to regulate tumorigenesis by its action on cancer cells and host stromal cells. We have recently reported that the metastasis of B16 melanoma into bone and other tissues including lung, liver and kidney was clearly suppressed in mPGES-1-deficient mice [10]. In these processes PGE2 produced by normal host cells, i.e. osteoblasts in bone and fibroblasts in soft tissues, was key to the regulation of both the growth and metastasis of the tumors [10]. Similar to the phenomena in mPGES-1-deficient mice, the administration of EP4 antagonist also abrogated the growth and metastasis of B16 melanoma in mice [10]. In the present study, we have shown that host osteoblasts produce PGE and express RANKL by an EP4-dependent mechanism that is initiated by contact with PC-3 cells, indicating that PGE-EP4 signaling is involved in the bone destruction and subsequent bone metastasis of prostate cancer PC-3 (Fig. 4B). The role of PGE-EP4 signaling in host cells may thus be a common pathway to regulate the microenvironment of various tumors, and EP4 antagonists may be a possible candidates for the prevention of bone metastasis.

In the treatment of prostate cancers that express the androgen receptor (AR) and grow in an androgen-dependent manner, the endocrine strategy is useful to induce androgen deficiency such as surgical castration and anti-androgen treatment [18]. After the treatment, however, recurrence of cancer cell growth occur as resistance to anti-androgen therapy [19]. In these advanced prostate cancer patients, bone metastases frequently occur and skeletal related events (SRE), including chronic pain, are closely related to the reduced quality of life in the patients [3, 5]. Using a xenograft model, Terada *et al.* [20] reported that the castration-resistant prostate cancer cells LNCap show the overexpression of EP4 in the cells, and that growth could be suppressed by EP4 antagonist *in vivo*, suggesting PGE signaling through EP4 in prostate cancer cells regulates the castration-resistant growth of prostate cancer. However neither LNCap or

PC-3 cell growth *in vitro* was affected by an EP4 antagonist [21], in agreement with the *in vitro* studies shown here (Fig. 3C). Since PGE acts on host stromal cells via EP4 receptor to regulate the growth and metastasis of prostate cancer, the effects of EP4 antagonist on the castration-resistance is not clear in prostate cancer.

In the present study, the cell-cell interactions between fixed prostate cancer cells and live osteoblasts elicits PGE2 production by osteoblasts, and stimulates RANKL expression which is suppressed by adding EP4 antagonist (Figs. 3A and 4A). Therefore, cell surface molecule(s) on PC-3 cells may induce PGE2 production in osteoblasts by cell-cell interactions. The integrin family are key molecules expressed at the cell surface and interact with other cells and ECM [22]. Regarding the mechanism of interaction between cancer cells and host cells, a possible role could be the tumor exosomes which are small membranous vesicles containing functional biomolecules (proteins, lipids, RNA and DNA) that can be horizontally transferred to recipient cells by membrane fusion [23, 24]. Recently Hoshino *et al.* [25] have reported that the integrin expression patterns in tumor exosomes is essential for pre-metastatic niche in the target tissues. Osteoblasts express a variety of integrin protein subunits, including β1, β3, α1, α2, α3, α4, α5, α6, and αv, and PC-3 also express several integrin molecules. In addition, osteoblasts actively produce ECM such as type I collagen which is a typical molecule bind to integrin. The roles of integrin molecules and tumor exosome in the microenvironment with bone metastasis of prostate cancer PC-3 remain to be examined in future studies.

In conclusion, the present study clearly showed that bone metastasis of human prostate cancer PC-3 was detected *in vivo* by the luminescence signals with soft-X ray in the experimental metastasis model of nude mice, and the bone metastasis with increased osteoclastic bone resorption was abrogated by the treatment of EP4 antagonist *in vivo*. The cell-to-cell interactions between PC-3 cells and host osteoblasts induced RANKL expression and PGE2 production by the osteoblasts, and adding EP4 antagonist suppressed the RANKL expression. Our data further define the mechanism by which the

blockade of PGE-EP4 signaling inhibits bone metastasis of prostate cancer. This strengthens the concept that EP4 antagonists could useful as therapeutic agents in advanced prostate cancer.

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Figure legends

Figure 1. The effect of EP4 antagonist on the metastasis of prostate cancer PC-3, and on the bone metastasis with increased osteoclastic bone resorption. (A) PC-3 cells expressing luciferase were injected into the left heart ventricle in nude mice, and some of the mice were orally administrated EP4 antagonist. On day 26, the metastasis of PC-3 was detected by the luminescence signal merged with the soft X-ray image. (B) Tibiae were collected from control mice, PC-3/vehicle mice, and PC-3/EP4 antagonist mice, and the sections were prepared for histological study in the proximal region of the tibiae. The sequential sections were stained by HE and TRAP, a specific marker enzyme for osteoclasts. Arrows indicate the growth area of PC-3 tumor, and the asterisk indicates the loss of cortical bone. Bar in TRAP staining is 300 μm. The tibiae were analyzed by 3D micro-CT. Bar in micro-CT is 2 mm.

Figure 2. The effect of EP4 antagonist on the bone distraction induced by bone metastasis of prostate cancer PC-3. (A) Three-dimensional images in micro-CT of the distal femurs collected from control mice, PC-3/vehicle mice, and PC-3/EP4 antagonist mice. (B) Bone morphometric analyses of the distal femur were performed using micro-CT to calculate BV/TV, BS/TS, Tb. Th and Tb. Sp. The data are expressed as the means ± SEM of control ($n = 5$), PC-3/vehicle ($n = 8$) or PC-3/EP4 antagonist ($n = 9$) mice. A significant difference between the two groups is indicated by: $^{*}p < 0.05$ *vs. control.* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, *vs. PC-3/vehicle.*

Figure 3. The production of PGE2 by osteoblasts and PC-3 cells induced by cell-to-cell interaction, and the effect of PGE and EP4 antagonist on the growth of PC-3 cells in vitro. (A) Mouse primary osteoblasts (POB) were cultured for 24 h with or without fixed-PC-3 cells. PC-3 cells were cultured for 24 h with or without fixed-POB. The concentrations

of PGE2 in each conditioned media were determined by EIA. The data are expressed as the means ± SEM of four independent wells. A significant difference between the two groups is indicated by: *$p < 0.001$ *vs. POB control.* (B) Expression of the PGE receptor subtype EP1-EP4 in PC-3 cells. The total RNA was extracted from PC-3 cells, and the expression of EP1, EP2, EP3 and EP4 mRNAs was detected by RT-PCR. (C) The effects of PGE2 and EP4 antagonist on the growth of PC-3 cells were examined by colony formation assay. PC-3 cells were cultured for 9 days with or without PGE2 (1 μM) and EP4 antagonist (10 μM), and the number of colonies was counted after crystal violet staining.

Figure 4. The expression of RANKL mRNA in osteoblasts induced by the interaction with fixed-PC-3. (A) Mouse primary osteoblasts were cultured for 24 h with fixed-PC-3 in the presence or absence of EP4 antagonist (10 μM). The total RNA was extracted, and the expression of RANKL mRNA was detected by RT-PCR. (B) The proposed mechanisms of bone resorption induced by prostate cancer PC-3 via PGE2/EP4 cascade.

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