Stimulation of Cyclooxygenase-2 Expression by Bone-Derived Transforming Growth Factor-β Enhances Bone Metastases in Breast Cancer

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Abstract

Cyclooxygenase-2 (COX-2), the rate-limiting enzyme of prostaglandin synthesis, has been implicated in invasiveness and distant metastases of cancer. Bone is one of the most common target sites of cancer metastasis. However, the role of COX-2 in bone metastasis is unclear. We examined the surgical specimens of bone metastases from patients with various types of cancers by using immunohistochemistry and observed evident COX-2 expression in these bone metastases. In a nude mouse model of bone metastasis, the MDA-MB-231 human breast cancer cells showed no COX-2 expression at orthotopic sites, whereas these cells, when metastasized to bone, intensely expressed COX-2, suggesting that the bone microenvironment induced COX-2 expression. Consistent with this notion, inhibition of bone resorption by the bisphosphonate ibandronate reduced COX-2 expression in MDA-MB-231 cells in bone. Transforming growth factor-β (TGFβ), one of the most abundant growth factors stored in bone, increased COX-2 expression and prostaglandin E2 production in MDA-MB-231 cells in culture. MDA-MB-231 cells overexpressing dominant-negative TGFβ type II receptors showed decreased bone metastases and reduced osteoclastic bone resorption with impaired COX-2 expression. The COX-2 inhibitors, NS-398 and nimesulide, significantly suppressed bone metastases with decreased osteoclast number and increased apoptosis in MDA-MB-231 cells. These results suggest that bone-derived TGFβ up-regulates COX-2 expression in breast cancer cells, thereby increasing prostaglandin E2 production, which in turn, stimulates osteoclastic bone destruction, leading to the progression of bone metastases. Our results also suggest that COX-2 is a potential therapeutic target for bone metastases in breast cancer. (Cancer Res 2006; 66(4): 2067-73)

Introduction

Cyclooxygenase (COX) is a rate-limiting enzyme of prostaglandin synthesis in the arachidonic acid cascade (1, 2). Cyclooxygenase has two isoforms, i.e., COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and is thought to be responsible for the production of prostaglandins that play roles in diverse physiologic functions. On the other hand, COX-2 is undetectable in the majority of normal tissues. However, it is rapidly induced by various stimuli and various pathologic conditions (1, 2). COX-2 has been implicated in cell growth, invasion, apoptosis, and angiogenesis in breast cancer (3–7). A substantial number of cases of human breast cancer have been found to express elevated COX-2, which was shown to be a negative prognostic factor in patients with breast cancer (8–10). Furthermore, epidemiologic studies have shown that the long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs), prototypic inhibitors of cyclooxygenase, reduces the incidence of breast cancer (11, 12). These results suggest the close relationship of COX-2 with the behaviors of breast cancer. One of the unique clinical features of breast cancer is that it has a strong predilection for spreading to bone (13). More than 70% of breast cancer patients at the terminal stages of the disease manifest bone metastases. To date, however, the role of COX-2 in bone metastasis of breast cancer has not been explored.

Meanwhile, it has been reported that prostaglandin production by human breast cancer cells positively correlates with the occurrence of bone metastases (14, 15), and that NSAIDs inhibit bone metastases of breast cancer (16). One of the COX-2 metabolites, prostaglandin E2 (PGE2), is known to stimulate osteoclastic bone resorption through the up-regulation of receptor activator of NFκB ligand (RANKL) in osteoblasts and/or bone marrow stromal cells (17–19). Osteoclastic bone resorption is a well-recognized key player in the development and progression of bone metastases (20–22). These findings collectively led us to hypothesize that COX-2 plays a contributive role in the bone metastasis of breast cancer.

In the present study, we examined this hypothesis using a well-characterized animal model in which inoculation of the MDA-MB-231 human breast cancer cells (MDA-MB-231 cells) into the left cardiac ventricle in female nude mice developed bone metastases. We also studied the mechanism underlying the regulation of COX-2 expression in MDA-MB-231 cells. Finally, the effects of selective COX-2 inhibitors, NS-398 and nimesulide, on bone metastases were determined. We found that bone-derived TGFβ up-regulated COX-2 expression in MDA-MB-231 cells and that the COX-2 inhibitors suppressed bone metastases of MDA-MB-231 breast cancer.

Materials and Methods

Reagents

Recombinant human transforming growth factor β1 (TGFβ1) was purchased from R&D Systems, Inc. (Minneapolis, MN). NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide), nimesulide [N-(4-nitro-2-phenoxypyphenyl)-methanesulfonyamide], and rabbit polyclonal antibodies to COX-1 and COX-2 were from Cayman Chemical Co. (Ann Arbor, MI). Rabbit polyclonal anti-phospho-Smad2 (Ser507/510) antibody was from Upstate Biotechnology (Lake Placid, NY). Goat polyclonal antibodies to Smad2 and actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Ibandronate [1-hydroxy-3-(methylpentylamino) propylidine

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biphasphonate] was provided by Boehringer Mannheim GmbH (Mannheim, Germany). All other chemicals used in this study were purchased from Sigma (St. Louis, MO) or Wako Pure Chemical Industries, Ltd. (Osaka, unless otherwise indicated.

### Surgical Specimens

Fifteen surgical specimens of bone metastases were studied. These specimens were obtained only from patients who had given informed consent and agreed to provide their tissues before surgery. The study protocol was approved by the Institutional Review Board of Osaka University Hospital. The clinical data are summarized in Fig. 1A.

### Animal Model of Bone Metastasis

Under anesthesia with pentobarbital (0.05 mg/g body weight; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), MDA-MB-231 cells were injected into the left cardiac ventricle (1 × 10⁶ cells/0.1 mL PBS) or the mammary fat pad (5 × 10⁶ cells/0.1 mL PBS) of 4-week-old female athymic nude mice (SLC Japan Co., Ltd., Hamamatsu, Japan) as previously described (23). Ibandronate (4 μg/mouse) was s.c. injected daily from days 21 to 28. NS-398 (20 mg/kg) or nimesulide (20 mg/kg) was i.p. injected daily from days 7 to 28. The number of mice used in each experiment was described in each figure. Mice were sacrificed at day 28. All animal experiments were reviewed by the Institutional Review Board of Animal Experiments at the Osaka University Graduate School of Dentistry.

### Radiographic Analysis

The number of osteolytic lesions was determined on radiographs at day 28 as described previously (23).

### Histologic and Histomorphometric Analysis

Histomorphometric analysis of tumor burden in bone was performed as described previously (23). Data are shown as tumor area (mm²)/animal.

#### Osteoclast number

For identification of osteoclasts, the sections were stained with TRAP as previously described (23). The number of TRAP-positive multinucleated osteoclasts at the interface between tumor and bone was counted in five fields of each section at ×400 magnification. Data are shown as the number of osteoclasts/mm² bone surface (N. Oc/mm²).

#### Apoptosis

Apoptosis in MDA-MB-231 cells was determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) technique using DeadEnd Colorimetric Apoptosis Detection System (Promega, Madison, WI) according to the manufacturer’s protocol. To determine the number of apoptotic cells, five fields of nonnecrotic areas of metastatic tumors in bone at ×400 magnification were randomly selected in each specimen and counted (23). Data are shown as the number of apoptotic cells/mm² tumor area.

### Immunohistochemistry for COX-2 Expression

All specimens were decalcified, embedded in paraffin, and processed by conventional histologic methods. Immunohistochemical staining of COX-2 was done using a rabbit polyclonal anti-COX-2 antibody and VECTASTAIN Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer’s protocol. Chromogen was developed using 3,3'-diaminobenzidine substrate kit (Vector Laboratories). The slides were counterstained with hematoxylin. The immunoreactivity was evaluated as negative (−) or positive (+). COX-2 expression of MDA-MB-231 tumors in nude mice was also determined by the same procedure as described above.

### Cell Culture

The estrogen receptor–negative human breast cancer cell line MDA-MB-231 (American Type Culture Collection, Rockville, MD) was cultured in DMEM (Sigma) supplemented with 10% FCS (Asahi Glass Techno Corp., Tokyo, Japan) and 100 μg/mL kanamycin sulfate (Meiji Seika Kaisha, Ltd., Tokyo, Japan). The mouse bone marrow stromal cell line, ST2, and the mouse osteoblastic cell line, MC3T3-E1, were cultured in α-MEM (Sigma) supplemented with 10% FCS and 100 μg/mL kanamycin sulfate. Mouse primary bone marrow stromal cells were isolated as described previously (24). All cells were maintained in a humidified atmosphere of 5% CO₂ in air.

### Transfection

A truncated TGFβ type II receptor construct lacking the cytoplasmic serine/threonine kinase domain was generated by PCR as described previously (25). The cDNA was tagged with Flag, subcloned into pcDNA3 vector (Invitrogen, San Diego, CA) and transfected into MDA-MB-231 cells using FuGENE 6 Transfection Reagent (Roche Diagnostics, Co., Indianapolis, IN) according to the manufacturer’s protocol (MDA/DN-T[RII]). As a control, empty pcDNA3 vector was similarly transfected into MDA-MB-231 cells (MDA/EV). Colonies resistant to 1 mg/mL G418 (Sigma) were isolated and cloned. The expression of the truncated TGFβ type II receptor in the selected clones was confirmed by Western blot as described below using anti-Flag monoclonal antibody (M2; Sigma).

### Immunoprecipitation

To quantitatively determine the COX-2 expression in vivo, metastatic tumors of MDA-MB-231 in bone were macroscopically dissected and
homogenized in lysis buffer [20 mMol/L HEPES (pH 7.4), 150 mMol/L NaCl, 1 mMol/L EGTA, 1.5 mMol/L MgCl₂, 10% glycerol, 1% Triton X-100, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mMol/L phenylmethylsulfonyl fluoride, 0.1 mMol/L sodium orthovanadate] using a tight-fitting Dounce homogenizer. The lysates were centrifuged for 15 minutes at 4°C at 16000 × g and incubated with anti-COX-2 antibody for 4 hours at 4°C, followed by immunoprecipitation with protein A agarose (Santa Cruz Biotechnology). Immunoprecipitates were washed five times with lysis buffer and boiled in SDS sample buffer, and supernatants were recovered as immunoprecipitate samples. The samples were then analyzed by Western blot as described below.

**Western Blot**

After overnight serum starvation, cells were treated with TGFβ (5 ng/mL) for 3, 6, 12, or 24 hours. The cells were then washed thrice with ice-cold PBS, and solubilized in the lysis buffer as described above. The cell lysates were boiled in SDS sample buffer containing 0.5 mol/L β-mercaptoethanol, separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted. Separated proteins were visualized with horseradish peroxidase coupled with protein A (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) with enhancement by chemiluminescence using ECL Western Blotting Detection Reagent (Amersham Bioscience Corp., Piscataway, NJ).

**RT-PCR**

MDA-MB-231 cells were plated in 10 cm culture dishes. After overnight serum starvation, the cells were treated with TGFβ (5 ng/mL) in the presence or absence of NS-398 (100 nMol/L-10 μMol/L) for 24 hours. After the incubation period, total RNA was isolated using TRI Reagent (Sigma). Single-stranded cDNA was synthesized from 3 μg of RNA using BD PowerScript Reverse Transcriptase (BD Biosciences, Palo Alto, CA). The primer sets used for PCR were as follows: human parathyroid hormone-related protein (PTH-rP), CAAGATTTACGGCGACGATT/GGGCTT-GCTTTTTCTTTCTTCT; human glyceraldehyde-3-phosphatase dehydrogenase (GAPDH), CATGGAGAAGGCCTGCT/C ACTGACACGTGCGATGG. PCR was done using a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems Japan, Ltd., Tokyo, Japan) under the following conditions: human PTH-rP, 94°C for 5 minutes, followed by 30 cycles at 94°C for 30 seconds, 58°C for 45 seconds, and 72°C for 1 minute; human GAPDH, 94°C for 5 minutes, followed by 25 cycles at 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 1 minute. The PCR products were separated on 2% agarose gels containing ethidium bromide and visualized under UV light. The sizes of the fragments were confirmed by reference to 100 bp DNA ladder. Quantification of amplified mRNA was done by densitometry assisted by the image analysis software Scion Image (Scion Corporation, Frederick, MD).

**ELISA**

MDA-MB-231 cells (1 × 10⁵) were plated in 48-well plates. When near confluence, the cells were rinsed with PBS, and 250 μL of serum-free DMEM with or without 5 ng/mL TGFβ and varying concentrations of NS-398 (10 μMol/L-100 μMol/L) was added to each well. The conditioned medium was collected after 48 hours. PGE₂ concentrations in the conditioned medium were determined by ELISA (Cayman Chemical) according to the manufacturer’s instructions. Data are expressed as the amount of PGE₂ produced (pg/mL) per 10⁵ cells.

**Cell Proliferation**

MDA-MB-231 cells (5,000 cells/well/96-well plate) were plated in DMEM supplemented with 10% FCS in the presence or absence of varying concentrations of NS-398 (1-100 μMol/L) and cultured for 48 hours. At the end of the culture, the cell proliferation was determined using Cell Proliferation Reagent WST-1 (Roche Diagnostics K.K., Tokyo, Japan) according to the manufacturer’s protocol. The absorbance was measured using a microplate reader (Model 550; Nippon Bio-Rad Laboratories, Tokyo, Japan) at a wavelength of 450 nm. The data were expressed as cell viability (% control) calculated with the following formula:

\[
\text{cell viability} = \left( \frac{\text{absorbance of treated cells}}{\text{absorbance of control cells}} \right) \times 100.
\]

**Apoptosis In vitro**

Subconfluent MDA-MB-231 cells in six-well plates were treated with NS-398 (1-100 μMol/L) for 48 hours. Apoptosis in MDA-MB-231 cells was determined using a fluorescence-activated cell sorter (FACS) technique as previously described (24). The percentage of sub-G₁ nuclei in the population was determined as the percentage of apoptosis.

**Statistical Analysis**

Data are expressed as the mean ± SE. The data were analyzed by one-way ANOVA followed by Student’s t test or Fisher’s PLSD post hoc test (StatView; SAS Institute, Inc., Cary, NC) for determination of differences between groups. Student’s t test or Welch's t test was conducted when two groups were compared. \( P < 0.05 \) was considered significant.

**Results**

**COX-2 expression in bone metastases of human cancers.**

Immunohistochemical examination showed that COX-2 was positive in cancer cells in bone metastases in 13 of 15 cases (86.7%; Fig. 1).

**COX-2 expression in MDA-MB-231 breast cancer.**

To investigate the relationship between COX-2 expression and bone metastases, we examined COX-2 expression in MDA-MB-231 cells in vivo by immunohistochemistry. MDA-MB-231 cells are derived from a human breast carcinoma, which is a well-known bone-seeking tumor (13), and we have reported that MDA-MB-231 cells reproducibly develop bone metastases in female nude mice (23). COX-2 was undetectable in MDA-MB-231 cells in the orthotopic mammary fat pad (Fig. 2A). In contrast, MDA-MB-231 cells in bone metastases, especially in the close vicinity of residual bone, expressed COX-2 (Fig. 2B). These results suggest that COX-2 expression in MDA-MB-231 cells is modulated by the local microenvironment. Because one notable feature of the bone microenvironment is continual osteoclastic bone resorption during bone remodeling, we reasoned that bone resorption regulates COX-2 expression in metastatic cancer cells. To explore this possibility, we examined the effects of the bisphosphonate ibandronate, a specific potent inhibitor of osteoclastic bone resorption (23), on COX-2 expression in MDA-MB-231 cells in bone metastases. We have previously shown that ibandronate inhibits osteoclastic bone resorption associated with bone metastases of MDA-MB-231 cells (23). Immunohistochemical and immunoprecipitation-Western analyses showed that ibandronate decreased COX-2 expression in metastatic MDA-MB-231 cells in bone compared with untreated mice (Fig. 2B and C). Western blot analysis showed that ibandronate did not reduce COX-2 expression in MDA-MB-231 cells in culture (Fig. 3C). These results suggest that the effects observed here were indirect due to the inhibition of osteoclastic bone resorption rather than direct suppression of COX-2 expression by ibandronate in MDA-MB-231 cells.

**Effects of TGFβ on COX-2 expression in MDA-MB-231.**

Evidence is accumulating that bone-stored growth factors are released into the bone microenvironment as a consequence of osteoclastic bone resorption and modulate the metabolic activity of cancer cells metastasized in bone (20, 22, 26, 27). The results shown in Fig. 2 suggest that COX-2 expression in MDA-MB-231 cells in bone is associated with osteoclastic bone resorption. Therefore, we examined whether bone-derived growth factors affect the COX-2 expression in MDA-MB-231 cells in vitro. Western blot analysis showed that TGFβ, one of the most abundant growth factors stored in bone (28, 29), increased COX-2 expression in MDA-MB-231 cells in a time-dependent manner (Fig. 3D). On the other hand, TGFβ...
did not affect COX-1 expression (Fig. 3A). These results suggest that the elevated COX-2 expression in MDA-MB-231 cells in bone metastases shown in Fig. 2 is, at least in part, due to TGFβ that is released following osteoclastic bone resorption. Of interest, we also found that TGFβ-induced COX-2 expression in mouse primary bone marrow stromal cells and osteoblastic cell lines, ST2 and MC3T3-E1 (Fig. 3B).

**Effects of DN-TβRII on COX-2 expression in MDA-MB-231 cells in bone metastases.** To further examine the role of bone-derived TGFβ on COX-2 expression in MDA-MB-231 cells in bone metastases, we established MDA-MB-231 cells stably transfected with truncated TGFβ type II receptor (MDA/DN-TβRII). This truncated receptor was shown to competitively block the ligand binding to the endogenous TGFβ type II receptor and inhibit TGFβ signaling activation in a dominant-negative fashion (25, 26). Western blot analysis showed that TGFβ-induced Smad2 phosphorylation was suppressed in MDA/DN-TβRII cells compared with MDA/EV cells (Fig. 4A, a), demonstrating that these receptors have dominant-negative effects in MDA-MB-231 cells. The introduction of DN-TβRII abolished the TGFβ-induced COX-2 expression in MDA-MB-231 cells (Fig. 4A, b). In addition, as previously described (26), TGFβ-induced PTH-rP expression was suppressed in MDA/DN-TβRII cells (Fig. 4B). Of importance, immunohistochemical examination revealed that COX-2 expression was markedly impaired in MDA/DN-TβRII cells in bone metastases compared with MDA/EV cells (Fig. 4C). Furthermore, consistent with the previous results reported by Yin et al. (26), the tumor burden of MDA/DN-TβRII cells in bone metastases was significantly smaller than MDA/EV cells (Fig. 4D).

**Role of bone-derived TGFβ in osteoclastogenesis associated with bone metastases.** In addition to decreased COX-2 expression in MDA/DN-TβRII cells, osteoclast number was also diminished in bone metastases of MDA/DN-TβRII cells (Fig. 5A), suggesting the role of bone-derived TGFβ in osteoclastogenesis associated with bone metastases. Our data showed that TGFβ significantly increased PGE2 production in MDA-MB-231 cells (Fig. 5B). PGE2 is a potent stimulator of osteoclast formation (17–19). TGFβ-increased PGE2 production was markedly decreased in the presence of a selective COX-2 inhibitor NS-398 in a dose-dependent manner (Fig. 5B). Consistent with these results, the conditioned medium harvested from TGFβ-treated MDA-MB-231 cultures significantly stimulated osteoclast-like cell formation in the mouse bone marrow cultures (data not shown). In contrast, the conditioned medium of MDA-MB-231 cells treated with TGFβ in the presence of NS-398 showed diminished effects on osteoclast-like cell formation (data not shown). These results suggest that bone-derived TGFβ increases PGE2 production through up-regulation of COX-2 expression in MDA-MB-231 cells, thereby stimulating osteoclastogenesis in bone metastases.

Because bone-derived TGFβ is shown to increase the production of another potent osteoclastogenic factor, PTH-rP, in MDA-MB-231 cells (26), we examined whether NS-398 affects TGFβ-induced PTH-rP expression in MDA-MB-231 cells. Semiquantitative RT-PCR analysis showed that NS-398 did not change TGFβ-increased PTH-rP mRNA expression (Fig. 5C), indicating that the action of NS-398 is specific for COX-2.

**Effects of COX-2 inhibitors on bone metastases of MDA-MB-231 cells.** To clarify the role of COX-2 in bone metastases, we subsequently examined the effects of COX-2 inhibitors on bone metastases of MDA-MB-231 cells. Radiographic analysis showed that the amount of metastases was significantly decreased in mice treated with NS-398 (data not shown). Histologic and histomorphometric examination showed that NS-398 significantly reduced the metastatic tumor burden of MDA-MB-231 cells (Fig. 6A and B). Consistent with our in vitro results, the number
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NS-398 significantly decreased viable cell number (Fig. 6f) and increased apoptosis (Fig. 6f) in a dose-dependent manner. These results suggest that COX-2 plays a role in cell proliferation and apoptosis in MDA-MB-231 cells.

Discussion

In the present study, we studied the role of COX-2 in bone metastases and the regulatory mechanism of COX-2 expression in breast cancer cells with a special focus on the relationship with the bone microenvironment. Our immunohistochemical study using surgical specimens showed that 87% of the surgical specimens of bone metastases of cancer patients expressed COX-2. Although the sample size we studied was not enough to draw a statistically significant conclusion, the high expression frequency of COX-2 in the bone metastases suggests a possible correlation between COX-2 expression and bone metastases. Animal experiments showed that MDA-MB-231 cells colonized in bone were positive for COX-2 using immunohistochemistry, and COX-2 expression seemed to be more intense in MDA-MB-231 cells in the close vicinity of the residual bone compared with cells distant from bone. Furthermore, the COX-2 expression in MDA-MB-231 cells in bone metastases was markedly decreased by the treatment with the bisphosphonate ibandronate. However, ibandronate did not directly reduce COX-2 expression in MDA-MB-231 cells in culture, suggesting that the decreased COX-2 expression by ibandronate treatment is due to the inhibition of osteoclastic bone resorption. Of note, COX-2 expression was undetectable in MDA-MB-231 cells in the mammary fat pad. These results suggest that COX-2 expression in MDA-MB-231 cells is under the influence of osteoclast bone resorption.
We next examined the effects of bone-stored growth factors (28, 29) that are released into the bone microenvironment following osteoclastic bone resorption on COX-2 expression in MDA-MB-231 cells. Accumulating evidence suggests that these bone-derived growth factors modulate the metabolic activity of cancer cells metastasized in bone (20, 22, 26, 27). Consistent with these notions, our data showed that TGFβ, which is stored in large amounts in bone, increased COX-2 expression in MDA-MB-231 cells in culture. More importantly, immunohistochemical study showed that the dominant-negative inhibition of TGFβ II receptor decreased COX-2 expression in MDA-MB-231 cells in bone metastases. Furthermore, our data also show that TGFβ up-regulates COX-2 expression in cells of osteoblast/stromal cell lineage. Ohshima et al. (30) showed that the coculture of MDA-MB-231 cells with osteoblasts increased COX-2 expression in osteoblasts, suggesting that COX-2 expressed in osteoblastic cells also plays a role in bone metastases. These results, together with the finding that ibandronate impaired COX-2 expression, suggest that bone-derived TGFβ induces COX-2 expression in MDA-MB-231 cells colonized in bone and resident osteoblasts/stromal cells.

It has been widely recognized that PGE2 stimulates osteoclastogenesis through the up-regulation of RANKL expression in osteoblasts/marrow stromal cells (17–19). We observed that the conditioned medium harvested from MDA-MB-231 cells treated with TGFβ significantly stimulated osteoclast-like cell formation in the mouse bone marrow cultures, suggesting that TGFβ induces osteoclastogenic factor production in MDA-MB-231 cells. This effect of TGFβ was reduced by the presence of a selective COX-2 inhibitor, NS-398, suggesting the involvement of prostaglandins. In support of this notion, TGFβ type II receptor decreased PGE2 expression, suggesting that TGFβ up-regulates COX-2 expression in osteoblast/stromal cell lineage.

Effects of the selective COX-2 inhibitors on bone metastases of MDA-MB-231 in nude mice and MDA-MB-231 cells in vitro. A, histologic view of bone metastases treated without (a) or with (b) NS-398. In untreated bone (a), trabecular bones are destroyed and the bone marrow cavity is replaced by metastatic MDA-MB-231 cells. In contrast, in NS-398-treated bone (b), colonization of metastatic MDA-MB-231 breast cancer cells is markedly decreased (BM, bone marrow; H&E staining, original magnification, ×40). B, histomorphometric analysis of tumor burden of MDA-MB-231 in the hind limbs treated with or without NS-398. Data are expressed as tumor area (mm2) per mouse (n = 10/group). *, P < 0.05, significantly different from untreated group. C, histologic view of TRAP staining of bone metastases in untreated (a) or NS-398-treated (b) mice. In untreated bone (a), there are numerous TRAP-positive osteoclasts along the bone surfaces (arrows). In contrast, osteoclast number is decreased in NS-398-treated bone (b; original magnification, ×200). D, histomorphometric analysis of osteoclast number in bone metastases treated with or without NS-398. Osteoclast number is expressed per mm of the tumor-bone interface (N. Oc/BS; n = 8/group). *, P < 0.01, significantly different from untreated group. E, histomorphometric analysis of tumor burden of MDA-MB-231 in the hind limbs treated with or without nimesulide (Nime). Data are expressed as tumor area (mm2) per mouse (n = 8/group). *, P < 0.05, significantly different from untreated group. F, histomorphometric analysis of osteoclast number in bone metastases treated with or without nimesulide (Nime). Osteoclast number is expressed per mm of the tumor-bone interface (N. Oc/BS; n = 8/group). *, P < 0.01, significantly different from untreated group. G, representative histologic view of TUNEL staining of bone metastases treated with NS-398. TUNEL-positive apoptotic MDA-MB-231 cells are clearly identified as brown-stained cells (arrows; original magnification, ×400). H, histomorphometric analysis of number of apoptosis in MDA-MB-231 cells in bone in untreated or NS-398-treated mice. Data are expressed as number of apoptosis per mm2 tumor area (n = 7/group). *, P < 0.01, significantly different from untreated group. I, effects of NS-398 on cell proliferation of MDA-MB-231 cells in vitro. Cell viability was determined using WST-1 as described in the text. Data are expressed as cell viability (% control; n = 4/group). *, P < 0.001, significantly different from control. J, effects of NS-398 on apoptosis of MDA-MB-231 cells in vitro. Apoptosis was determined by FACS. The percentages of sub-G1 nuclei in the population were determined as the percentage of apoptosis (n = 4/group). *, P < 0.001, significantly different from control.
lower than MDA/EV cells. Collectively, these results suggest that bone-derived TGFβ promotes osteolysis and bone resorption through the induction of COX-2 expression and consequent PGE2 production in MDA-MB-231 cells, which in turn, up-regulates RANKL expression in osteoblasts/stromal cells. Thus, COX-2 plays a critical role in the promotion of osteolytic bone metastases by stimulating osteoclastic bone resorption.

In addition to the stimulation of COX-2 expression and PGE2 production, bone-derived TGFβ is also shown to increase the production of PTHrP, a potent stimulator of osteolysis in MDA-MB-231 cells, and contributes to the promotion of osteolytic bone metastases by mediating the establishment of a vicious cycle between breast cancer cells and osteoclastic bone resorption (20, 22, 26). Indeed, our RT-PCR analysis showed that TGFβ increased PTHrP mRNA expression in MDA-MB-231 cells. Moreover, inhibition of bone metastases and osteoclastic bone resorption by NS-398 and nimesulide in vitro was partial, suggesting the involvement of osteoclastogenic factors other than prostaglandins. Taken together, it is suggested that bone-derived TGFβ plays a critical role in promoting osteolytic bone metastases by up-regulating the production of both prostaglandins and PTHrP in breast cancer cells.

Previous studies have shown that selective COX-2 inhibitors inhibit cell growth and induce apoptosis in breast cancer cells (4, 5). Consistent with these results, our in vitro studies showed that NS-398 decreased cell proliferation and increased apoptosis in MDA-MB-231 cells. Furthermore, our histomorphometric examination showed that NS-398 increased apoptosis in MDA-MB-231 cells in bone metastases. These results suggest that COX-2 inhibitors possess direct anticancer effects on breast cancer cells.

We propose that COX-2 expression in breast cancer cells contributes to the development and progression of bone metastases through the following mechanism. Osteoclastic bone resorption releases TGFβ from the bone matrix, leading to increased COX-2 expression, and consequently, PGE2 production in breast cancer cells colonizes the bone. PGE2 then up-regulates RANKL in osteoblasts/marrow stromal cells, which in turn, stimulates osteoclastic bone resorption, thereby establishing the vicious cycle between osteoclasts and breast cancer cells. COX-2 inhibitors can partially reduce osteolytic bone metastases by disrupting this vicious cycle and directly inhibiting cell growth and inducing apoptosis in breast cancer cells.

In conclusion, our results suggest that COX-2 expression regulated by bone-derived TGFβ in breast cancer cells plays an important role in osteolytic bone metastasis. The results also suggest that COX-2 is a potential therapeutic target in designing pharmacologic interventions for bone metastases in breast cancer.

References
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