Bone-Derived IGF Mediates Crosstalk between Bone and Breast Cancer Cells in Bony Metastases

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Introduction

Bone is one of the most common target sites of distant metastasis of breast, prostate, and lung cancer (1–3). Bone metastasis represents one of the major causes of morbidity such as intractable bone pain, pathologic fractures, spinal cord compression, and hypercalcemia in cancer patients. Accumulated clinical and experimental data that specific inhibitors of osteoclasts including bisphosphonates (BP) effectively suppress osteolytic bone metastases (4, 5) suggest that osteoclastic bone destruction plays a critical role in the development and progression of bone metastases.

Abstract

The continuous release of bone-stored growth factors after bone resorption promotes the colonization of circulating cancer cells. However, the precise role of each of the various growth factors remains unclear. In this study, we investigated the role of bone-derived insulin-like growth factor (IGF) in the development of bone metastases in an animal model of breast cancer. We found that local stimulation of calvarial bone resorption before cell inoculation stimulated subsequent bone metastases to that site in vivo, although inhibition of bone resorption inhibited bone metastases. Anchoroge-independent growth of cancer cells was stimulated by the culture supernatants from resorbed bones, which contained elevated levels of IGF-I. This stimulation was blocked by IGF type I receptor (IGF-IR) neutralizing antibody, but not antibody targeting other bone-stored growth factors including TGF-β, fibroblast growth factors, and platelet-derived growth factors. Although recombinant human IGF-I caused IGF-IR tyrosine autophosphorylation, followed by activation of Akt and NF-kB in cancer cells, dominant-negative inhibition of IGF-IR, Akt, or NF-kB significantly reduced bone metastases with increased apoptosis and decreased mitosis in metastatic cells. Together, our findings suggest that bone-derived IGF-I bridges the crosstalk between bone and metastasized cancer cells via activation of the IGF-IR/Akt/NF-kB pathway. Disruption of this pathway therefore may represent a promising therapeutic intervention for bone metastasis. Cancer Res; 72(16): 1–12. ©2012 AACR.

Bone is a storehouse of a variety of growth factors such as insulin-like growth factors (IGF), TGF-β, fibroblast growth factors (FGF), platelet-derived growth factors (PDGF), and bone morphogenetic proteins (BMP; refs. 6 and 7). Our group showed that TGF-β is released in active forms after bone resorption in organ cultures of neonatal mouse calvarial bones (8), verifying that bone-stored growth factors are released by osteoclastic bone resorption.

Several lines of evidence suggest that colonization of cancer cells in bone is under the influence of bone-derived growth factors. For example, bone-derived TGF-β has been shown to activate TGF-β-Smad signaling pathway in metastatic breast cancer cells in bone leading to increased parathyroid hormone-related protein (PTHrP) production in these breast cancer cells (9, 10), which in turn enhances osteoclastogenesis and bone destruction through an upregulation of receptor activator of NF-kB ligand (RANKL) expression in neighboring osteoblasts (11). RANKL in osteoblasts then functionally interacts with RANK expressed in preosteoclasts and mature osteoclasts, causing increased osteoclast formation and bone resorption. Consequently, growth factors are further released from resorbing bone and promote colonization of metastatic cancer cells in bone. Accordingly, it has been proposed that an establishment of so-called “vicious cycle” between metastatic cancer cells and bone is a requisite for the development of bone metastases (1–3). However, the contributions of bone-stored growth factors other than TGF-β to bone metastasis are still poorly understood. Moreover, dissection of cancer cell responses to these bone-derived growth factors in the
development of bone metastases is important to understand the mechanism of bone metastases and to design novel therapeutic approaches for bone metastases.

IGFs, which are the most abundant growth factors stored in bone (6, 7), have been implicated in the development, progression, and aggressiveness of many types of cancers including breast cancer (12, 13). Therefore, the role of IGFs in bone metastasis is of particular interest and worthwhile studying. IGFs initiate its actions primarily through binding to IGF type I receptors (IGF-IR; refs. 12 and 13). Clinical studies described that the majority of cancers express IGF-IR (14) and that the expression levels are significantly higher in tumors than neighboring normal tissues (15). Experimental studies showed that the activation of IGF-IR signaling pathways promotes cancer growth and metastasis (16). Conversely, inhibition of IGF-IR signaling suppressed tumor aggressiveness (17). These results suggest that IGF-IR expression and activation of its signaling pathway are critical to the promotion of malignant behaviors of cancers. Accordingly, IGF-IR and its downstream pathway have been thought to be promising targets for cancer therapy and varieties of antagonists, inhibitors or neutralizing antibodies are currently under clinical evaluation (12, 13).

In this study, we attempted to determine the role of bone-derived IGFs and IGF-IR signaling pathway in responding breast cancer cells in the development of bone metastases in a preclinical setting using a well-characterized animal model (4). We found that the bone-derived IGFs stimulated bone metastases of MDA-MB-231 human breast cancer cells through stimulation of cell proliferation and inhibition of apoptosis. These effects of IGFs were elicited via the activation of the serine/threonine kinase Akt and the transcription factor NF-κB. These effects of IGFs were elicited via the activation of Akt and NF-κB (12, 13, 18–21). Disruption of the activation of IGF-IR, Akt, or NF-κB significantly inhibited bone metastases. Our results suggest that an activation of IGF-IR signaling by bone-derived IGFs promotes bone metastases of breast cancer. IGF-IR signaling pathway could thus be a well-rationaled target in the development of pharmacological therapeutic agents for bone metastasis.

Materials and Methods

Cytokines and antibodies

Recombinant human TGF-β1, IGF-I, IGF-II, FGF-1, FGF-2, BMP-2, PDGF-BB, and interleukin-1β (IL-1β), and neutralizing polyclonal antibodies to TGF-β, FGF-1, FGF-2, and PDGF-BB were purchased from R&D Systems. A neutralizing mouse monoclonal antibody to IGF-IR (αIGFIR) was from Oncogene Research Products. Rabbit polyclonal antibodies to IGF-IRα and NF-κB (p50 and p65) were from Santa Cruz Biotechnology. Rabbit polyclonal antibodies to Akt and phospho-Akt (pAkt) were from Cell Signaling Technology. Anti-phosphotyrosine (pY) polyclonal antibody was described previously (22). The BP zoledronic acid (ZOL) was from Novartis Pharma. All other chemicals used in this study were purchased from Sigma-Aldrich or Wako Pure Chemical Industries unless otherwise described.

Cell culture

Human breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection. The cells were expanded and stored according to the supplier’s instructions, and used within 2 months after resuscitation of frozen aliquots. MDA-231AD cells, a MDA-MB-231 clone that is reproducibly and highly metastatic to bone and adrenal glands, was described previously (23). These cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS (Hyclone) and 1% penicillin–streptomycin solution (Life Technologies) in a humidified atmosphere of 5% CO2 in air.

Stimulation of calvarial bone resorption in organ culture

The details of this culture technique have been described previously (24). In brief, calvariae were excised from 6-day-old pups of BALB/c mice (Harlan), dissected free of adjacent connective tissues, placed in serum-free Biggers-Gwatkin-Jackson medium (BGJb; Sigma-Aldrich) containing 0.1% BSA and cultured with or without 100 pmol/L IL-1b for 48 hours in the absence or presence of 1 μmol/L ZOL. The conditioned medium (CM) harvested from IL-1β-treated or -untreated calvarial bones was designated resorbed or unresorbed bone CM, respectively and frozen at −20°C until use.

Colony formation assay

Anchorage-independent growth of MDA-MB-231 cells was determined by colony formation in soft agar as described (25). MDA-MB-231 cells (500 cells/well/24-well plate) were cultured in DMEM supplemented with 2% FBS and control bone or resorbed bone CM (20%, v/v) in the absence or presence of 5 μg/ml neutralizing antibodies to TGF-β, IGF-IR, FGF-1, FGF-2, or PDGF-BB in soft agar for 14 days. CM and antibodies were added to the cultures every 3 and 7 days, respectively. At the end of culture, colonies >200 μm in diameter were manually counted under inverted microscope.

Measurements of PTHrP and IGF-1

PTHrP production by MDA-MB-231 clones was measured as described (9). PTHrP concentrations were determined using a 2-site immunoradiometric assay (IRMA; Nichols Institute or Mitsubishi Chemical) according to manufacturer’s instruction. IGF-1 concentrations were measured using a commercial RIA kit (American Laboratory Products Company).

Transfection

Cell transfection was carried out as described in Supplementary Materials and Methods. Wild-type IGF-IR gene was stably transfected into parental MDA-MB-231 cells and dominant-negative mutants of IGF-IR (IGF-IR/486STOP), Akt (DN-Akt), and inhibitor of xBtx (IxBtxAN) were into MDA-231AD cells.

Tumor inoculation

Tumor cell inoculation was carried out as described in Supplementary Materials and Methods. All animal protocols were approved by the Institutional Animal Care and Use...
Committee at Osaka University Graduate School of Dentistry and the University of Texas Health Science Center at San Antonio.

Radiographic analysis
Development of bone metastases was monitored by X-rays as described previously (4).

Histological and histomorphometric analysis
Paraffin sections of the hindlimbs were made after conventional methods. Histomorphometric analyses of metastatic tumor burden in bone, apoptosis, and mitosis of MDA-MB-231 cells, and osteoclast number in bone metastases were carried out as described previously (4).

IGF-IR immunohistochemistry
Fifteen clinical samples of bone metastases were obtained at the time of surgery at Osaka University Hospital (Osaka, Japan). The study was approved by the Institutional Review Board of the Osaka University Graduate School of Medicine. Immunohistochemical staining of paraffin sections was carried out using Histofine Simple Stain Kit (Nichirei Biosciences) according to the manufacturer’s protocol. Chromogen was developed using DAB Liquid System (Dako). The slides were counterstained with hematoxylin. The immunoreactivity was evaluated as negative (−) or positive (+).

Immunoprecipitation and Western blotting
Immunoprecipitation (IP) and Western blotting (WB) were carried out as described previously (26).

Electrophoretic mobility shift assay
Electrophoretic mobility shift assay (EMSA) was carried out as described in Supplementary Materials and Methods.

Statistical analysis
Data are expressed as the mean ± SEM. The data were analyzed by 1-way ANOVA followed by Fisher’s PLSD post hoc test (StatView; SAS Institute, Cary, NC) for determination of differences between groups. Student t test or Welch t test was conducted when 2 groups were compared. P values of <0.05 were considered significant.

Results
Effects of bone resorption on the development of bone metastases
We previously described that MDA-MB-231 cells rarely metastasized to calvarial bones after intracardiac inoculation for unknown reasons (7). Taking advantage of this feature, we examined whether stimulation of calvarial bone resorption in advance modulates the subsequent development of bone metastases to this rare site. Bone resorption was stimulated by the repeated subcutaneous injections of IL-1β over the calvariae (Fig. 1A). This treatment induced no evident stimulation of bone resorption in other bones than calvariae at radiologic and histologic levels and did not cause hypercalcemia (27). Thus, the effects of IL-1β were restricted to the calvarial bones. Twenty-four hours after the last injection of IL-1β, MDA-MB-231 cells were inoculated into the left cardiac ventricle in female nude mice (Fig. 1A). At day 28, mice treated with IL-1β before cell inoculation showed macroscopic tumor formation on the calvariae (Fig. 1B). Radiographic examination revealed multiple osteolytic lesions on the calvarial bones (Fig. 1C). Quantitative assessment of these osteolytic lesions showed that stimulation of calvarial bone resorption by IL-1β before to cell inoculation significantly increased osteolytic area (Fig. 1C). Histologic examination showed that these osteolytic lesions were colonized by metastatic cancer cells with destruction of calvarial bones by numerous osteoclasts (Fig. 1D), verifying that these osteolytic lesions represent bone metastases. Mice received repeated IL-1β injections on their calvarial bone but no subsequent intracardiac inoculation of cancer cells exhibited no development of osteolytic lesions at day 28 (data not shown). However, we observed discernible increase in calvarial bone formation probably as a consequence of IL-1β-stimulated bone resorption as described in the previous report (27). IL-1β administered together with the BP ZOL, a potent and specific inhibitor of osteoclastic bone resorption (5), showed significant reduction of osteolytic bone metastases on calvarial bones (Fig. 1C). ZOL alone with no IL-1β injections, followed by intracardiac inoculation of MDA-MB-231 cells showed no effects on calvarial bones, since no osteolytic bone metastases were developed on the calvarial bones in the absence of IL-1β injections. However, under these circumstances, osteolytic bone metastases developed in the proximal tibiae that are one of the representative sites of bone metastasis in this animal model and zoledronic acid significantly reduced these osteolytic bone metastases (data not shown). These results suggest that the stimulation of bone resorption stimulates bone metastases, whereas inhibition of bone resorption inhibits them.

Effects of resorbed bone CM on MDA-MB-231 cells
We next attempted to identify a bone-derived factor that is released after stimulation of bone resorption and is responsible for stimulation of bone metastases. To study this, neonatal mouse calvarial bones were placed in organ cultures in the presence or absence of IL-1β with or without ZOL, and the CM was harvested and tested for their capacity to stimulate MDA-MB-231 cell behaviors. Earlier studies from our group reported that bone resorption of neonatal mouse calvariae was stimulated under this experimental condition (24) and that bone-stored growth factors are released in the culture medium in active forms (8).

To examine the effects of the CM on cell growth, we carried out the colony formation assay in soft agar. This assay has been widely used for determining in vitro tumorigenicity of cancer cells (28) and shown to correlate well with in vivo tumorigenicity. The resorbed bone CM (20%, v/v) markedly stimulated the anchorage-independent growth of MDA-MB-231 cells in soft agar compared with the control bone CM (Fig. 2A). The effects of the resorbed bone CM were dose-dependent between concentrations of 10% to 50% (v/v; data not shown). The CM harvested from the cultures treated with both IL-1β and ZOL showed profoundly reduced activity compared with those
treated with IL-1β alone (Fig. 2A). Of note, the neutralizing antibody to IGF-IR but not TGF-β, FGF-1, FGF-2, and PDGF-BB significantly inhibited the colony formation that was stimulated by the resorbed bone CM (Fig. 2B). In separate experiments, we found that 5 μg/mL TGF-β, IGF-IR, FGF-1, FGF-2, or PDGF-BB antibodies could neutralize growth-modulating activity of 10 ng/mL TGF-β, 100 ng/mL IGF-IR, 25 ng/mL FGF-1, 25 ng/mL FGF-2, and 25 ng/mL PDGF-BB, respectively (data not shown). The concentrations of IGF-1 were significantly greater in the resorbed bone CM than control bone CM and were significantly decreased in the presence of ZOL (Fig. 2C). Furthermore, recombinant human IGFs showed the greatest dose-dependent stimulation of the colony formation among the growth factors tested (Fig. 2D). These results collectively suggest that IGFs released from bone as a consequence of bone resorption are responsible for the promotion of anchorage-independent growth in MDA-MB-231 breast cancer cells.

Figure 1. Effects of bone resorption on the development of bone metastases of MDA-MB-231 human breast cancer cells in nude mice. A, experimental protocol. Four-week-old female athymic nude mice received repeated subcutaneous injections of IL-1β (thin arrows, 2 μg/10 μL/injection) over the calvariae 3 times a day for 3 days. Twenty-four hours after the last injection, MDA-MB-231 cells were inoculated into the left cardiac ventricle (thick arrow). In one group of animals, ZOL (4 μg/mouse, s. c., daily) was given from 4 days before the IL-1β injection to the end of the experiments. Mice were sacrificed at day 28. B, macroscopic view of tumor formation at the time of sacrifice. Note tumor formation on the head of IL-1β-treated mouse (arrow). C, representative radiographic view of calvariae and quantitative analysis of osteolytic lesions in PBS-, IL-1β-, and IL-1β + ZOL-treated mice. Data are mean from 2 separate experiments (n = 6 mice × 2 experiments = 12 for each group). *, significantly greater than PBS group (P < 0.01). †, significantly less than IL-1β group (P < 0.01). D (left), representative histologic view of metastasis in calvariae; higher magnification of the dashed rectangle area (right). Tumor colonization (T) is associated with calvarial bone destruction with numerous TRAP-positive osteoclasts along the surface of adjacent bone [TRAP staining; Br, brain; scale bars, 500 μm (left) and 100 μm (right)].
Role of IGF/IGF-IR in bone metastases of MDA-MB-231 cells

To examine the role of IGFs in the development of bone metastases in vivo, we first established 2 MDA-MB-231 clones (Cl. 1 and Cl. 2) stably transfected with the dominant-negative IGF-IR (MDA/486STOP). In MDA/486STOP cells, expression of the endogenous IGF-IR was not altered compared with the empty vector-transfected cells (MDA/EV; Fig. 3A, bottom left, thin arrow); however, tyrosine autophosphorylation of IGF-IR induced by IGF-I was almost abolished (Fig. 3A, top left) in conjunction with the expression of large amounts of the dominant-negative IGF-IR (IGF-IR/486STOP; Fig. 3A, bottom left, thick arrow). IGF-IR/486STOP was secreted into the culture medium from MDA/486STOP cells because of a lack of transmembrane domain (Fig. 3A, right), which competitively inhibited the binding of IGFs to the endogenous IGF-IR (17).

We then examined the capacity of MDA/486STOP cells to develop bone metastases. Radiographic analysis showed that the development of osteolytic lesions was markedly suppressed in MDA/486STOP (Fig. 3B). Histologic examination also showed that the metastatic tumor burden of MDA/486STOP in bone was significantly reduced compared with MDA/EV (Fig. 3C). Osteoclast number was not different between the groups (data not shown). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining revealed that apoptosis was increased in MDA/486STOP (Fig. 3D and E), whereas cell mitosis was significantly decreased (Fig. 3F). In addition, IL-1β–induced metastases to calvarial bones, as determined according to the same method as Fig. 1A, were markedly reduced in MDA/486STOP (Fig. 3G).

These results suggest that IGF-IR activation by bone-derived IGF facilitates the development of bone metastases by decreasing apoptosis and increasing proliferation of cancer cells colonizing bone.

To determine whether these effects of IGF/IGF-IR are specific for bone metastases, tumor growth in the orthotopic mammary fat pad, and the capacity to develop lung metastases were examined. Tumor growth of MDA/486STOP in the orthotopic site was not different from that of MDA/EV (Fig. 3H). Lung metastases were marginally reduced in MDA/486STOP (Fig. 3I).

Because IGF-IR/486STOP is a secreted form of IGF-IR, the possibility remains that it decreases bone metastasis via inhibition of IGF-IR signaling not only in MDA-MB-231 cells but also other neighboring bone-resident cells. To exclude this, we established MDA-MB-231 clones in which IGF-IR was stably knocked down (MDA/shIGF-IR) and tested their ability to develop bone metastases. Consistent with the results of
MDA/486STOP, IGF-IR knockdown also significantly reduced bone metastases (Supplementary Fig. S1), suggesting the primary role of IGF-IR signals in cancer cells in the development of bone metastasis.

As an alternative approach to verify the involvement of bone-derived IGF and IGF-IR activation in bone metastasis, we carried out experiments using MDA-MB-231 cells over-expressing wild-type IGF-IR (MDA/IGF-IR). The expression of IGF-IR protein (Fig. 4A, bottom) and its tyrosine autophosphorylation induced by IGF-I (Fig. 4A, top) were markedly increased in MDA/IGF-IR compared with MDA/EV. MDA/IGF-IR showed marked increases in osteolytic lesions (Fig. 4B, E, F).
n EV and cancer cells in bone metastases in patients with several types of cancers. Immunohistochemical study revealed that 13 of 15 cases (86.7%; Fig. 5A) showed strong expression of IGF-IR (Fig. 5B) in cancer cells colonizing bone, suggesting an important role of IGF/IGF-IR axis in bone metastases in cancer patients regardless of the primary site of tumors.

**Role of Akt in bone metastases of MDA-MB-231 cells**

Because Fig. 3D and E show that increased apoptosis in MDA/486STOP is associated with reduced bone metastases, we next studied the involvement of the serine/threonine kinase Akt in the development of bone metastases. Akt is a downstream molecule of IGF-IR signaling and is widely recognized as a cell survival or an antiapoptotic factor (12, 13, 21). Western

### Figure 4. Effects of wild-type IGF-IR overexpression in MDA-MB-231 cells on bone metastases.

A, tyrosine phosphorylation and expression of IGF-IR in MDA/EV (EV) and MDA/IGF-IR (IGF-IR) determined by immunoprecipitation (IP)-Western analysis. After overnight serum starvation, cells were treated with or without 100 ng/mL IGF-I for 10 minutes. B and C, radiologic analysis of osteolytic lesions of MDA/EV and MDA/IGF-IR in the hindlimbs (arrows: osteolytic lesions, n = 5 for EV and n = 7 for IGF-IR clones).

D, histomorphometric analysis of tumor burden of MDA/EV and MDA/IGF-IR in the hindlimbs (n = 5 for EV and n = 7 for IGF-IR clones). E, tumor development of MDA/EV and MDA/IGF-IR in the orthotopic mammary fat pad in nude mice (n = 3/group).

C, D, *: significantly different from EV (P < 0.05).

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### IGF-IR expression in bone metastases in cancer patients

To explore the clinical relevance of these findings obtained in preclinical settings, we examined the expression of IGF-IR in cancer cells in bone metastases in patients with several types of cancers. Immunohistochemical study revealed that 13 of 15 cases (86.7%; Fig. 5A) showed strong expression of IGF-IR (Fig. 5B) in cancer cells colonizing bone, suggesting an important role of IGF/IGF-IR axis in bone metastases in cancer patients regardless of the primary site of tumors.
sine phosphorylation of insulin receptor substrate-1 (IRS-1) was not changed (Fig. 6C).

The number of apoptosis in bone determined by TUNEL staining was compared with MDA/EV (Fig. 6B). The number of apoptosis in MDA/DN-Akt cells in bone metastases was significantly reduced compared with MDA/EV, whereas mitosis in MDA/DN-Akt was significantly decreased (Fig. 7C). Apoptosis in MDA/IκBα cells in bone metastases was significantly increased compared with MDA/EV cells (Fig. 7C), whereas mitosis in MDA/IκBα was significantly decreased (Fig. 7C). Osteoclast number at tumor–bone interface was decreased in bone metastases of MDA/IκBα (Fig. 7C). Of note, however, production of PTHrP, a potent stimulator of osteoclastogenesis, in MDA/IκBα cells in the absence or presence of TGF-β was not changed compared with MDA/EV cells (Fig. 7D).

**Figure 6. Role of Akt signaling in bone metastases of MDA-MB-231.**

A, top, IGF-I-induced Akt phosphorylation at threonine 308 in MDA/486STOP (486STOP; left) and MDA/IGF-IR (IGF-IR; right) determined by Western blot. After overnight serum starvation, cells were treated with or without 100 ng/ml IGF-I for 10 minutes; bottom, expression of Akt protein determined by Western blot. B, histologic view (left) and histomorphometric analysis (right) of bone metastases of MDA-MB-231 cells stably transfected with dominant-negative Akt (MDA/DN-Akt) in nude mice [n = 15 for MDA/EV (EV) and n = 14 for MDA/DN-Akt (DN-Akt); H&E staining; T, tumor; BM, bone marrow; scale bar, 500 μm]. C, histomorphometric analysis of number of apoptosis and mitosis in MDA/EV and MDA/DN-Akt cells in bone metastases (n = 9 for MDA/EV and n = 7 for MDA/DN-Akt). B and C, *p < 0.05.

Western blot analysis showed that IGF-I induced phosphorylation at threonine 308 (Fig. 6A, top left) and serine 473 (data not shown) of Akt in MDA/EV. Phosphorylation at threonine 308 was abolished in MDA/486STOP (Fig. 6A, top left) and increased in MDA/IGF-IR (Fig. 6A, top right). These results show that IGF-I/IGF-IR axis activated Akt. To examine the role of Akt activation in bone metastasis, we established MDA-MB-231 cells expressing dominant-negative Akt (MDA/DN-Akt). Histologic and histomorphometric analysis showed that tumor burden in bone was significantly reduced in MDA/DN-Akt compared with MDA/EV (Fig. 6B). The number of apoptosis in MDA/DN-Akt cells in bone determined by TUNEL staining was significantly increased, although the number of mitotic cells was not changed (Fig. 6C).

In separate experiments, we observed that IGF-I caused tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and p85 phosphatidylinositol 3-kinase (PI3K; ref. 29), leading to a complex formation of IRS-1 with p85 PI3K upstream of Akt activation. Thus, the conventional IGF-I/IGF-IR signaling pathway is propagated in MDA-MB-231 human breast cancer cells.

**Role of NF-κB in bone metastases of MDA-MB-231 cells**

As another downstream molecule of IGF-IR signaling whose activation is associated with survival of cancer cells, we examined the role of the transcription factor NF-κB in bone metastasis. EMSA analysis showed that IGF-I activated the p50 and p65 subunit of NF-κB in MDA/EV (Fig. 7A, left and center). MDA/IGF-IR exhibited increased NF-κB activation (Fig. 7A, left). IGF-I failed to activate NF-κB in MDA/486STOP cells (data not shown). NF-κB activation was also markedly suppressed in MDA-MB-231 cells expressing dominant-negative IκBα (MDA/IκBαDN; Fig. 7A, right). Histologic and histomorphometric analysis revealed that MDA/IκBαDN developed significantly reduced bone metastases (Fig. 7B). Apoptosis in MDA/IκBαDN cells in bone metastases was significantly increased compared with MDA/EV cells (Fig. 7C), whereas mitosis in MDA/IκBαDN was significantly decreased (Fig. 7C). Osteoclast number at tumor–bone interface was decreased in bone metastases of MDA/IκBαDN (Fig. 7C). Of note, however, production of PTHrP, a potent stimulator of osteoclastogenesis, in MDA/IκBαDN cells in the absence or presence of TGF-β was not changed compared with MDA/EV cells (Fig. 7D).

**Discussion**

Bone is one of the most preferential target organs of cancer metastasis (1–3). Although the precise mechanism by which cancer cells preferentially spread to bone has not been fully understood, it has been suggested that bone-derived growth factors create favorable microenvironment for cancer cells to survive and colonize bone. Consistent with this notion, our in vivo study showed that stimulation of osteoclastic bone resorption in calvarial bones by repeated injections of IL-1β before cell inoculation markedly increased subsequent MDA-MB-231 breast cancer cell metastases to that local site. We previously described that MDA-MB-231 cells rarely metastasize to calvarial bones following intracardiac inoculation in this model for unknown reasons (7). In contrast, inhibition of bone resorption by cotreatment with IL-1β and the BP ZOL, a potent inhibitor of osteoclastic bone resorption, significantly decreased MDA-MB-231 metastases to calvarial bones. The CM harvested from resorbed bones increased anchorage-independent growth of MDA-MB-231 cells in soft agar compared with that of control bone. On the other hand, the anchorage-independent growth was not stimulated by the CM harvested from bones treated with IL-1β together with ZOL. We previously described that bone-stored growth factors are released from resorbed bone into the culture medium in active forms (8). Taken together, these results suggest that stimulation of bone resorption can induce bone metastases at this rare site via promotion of breast cancer cell colonization because of increased supply of bone-stored growth factors. Our experiments prove that cancer metastasis to bone is dependent on the extent of osteoclastic bone resorption.
Figure 7. Role of NF-κB signaling in bone metastases of MDA-MB-231. A, IGF-I-induced activation of NF-κB in MDA/IGF-IR (IGF-IR, left) and MDA/IκBαΔN (ΔN, right) determined by EMSA (EV: MDA/EV). After overnight serum starvation, cells were treated with or without 500 ng/mL IGF-I for 4 hours. Thick arrows indicate specific bands for NF-κB. The specificity was confirmed by supershift assay using antibodies to NF-κB subunits p50 and p65 (center, thin arrows). B, histologic analysis of bone metastases of MDA/EV and MDA/IκBαΔN (IκBαΔN or ΔN; n = 6 for EV and n = 5 for IκBαΔN). H&E staining; T, tumor; BM, bone marrow; scale bar, 500 μm. C, histomorphometric analysis of apoptosis and mitosis in MDA/EV and MDA/IκBαΔN in culture in the presence or absence of TGF-β (5 ng/mL) (n = 4/group). B and C, *, significantly different from EV (P < 0.05). D, **, significantly different from EV in the absence of TGF-β (P < 0.01).

Next we studied which bone-stored growth factor is responsible for promotion of anchorage-independent growth of MDA-MB-231 cells. To approach this, the effects of the neutralizing antibodies to the growth factors that are known to be stored in bone (6, 7) were examined in colony formation assay. Notably, anchorage-independent growth-stimulatory effects of the resorbed bone CM on MDA-MB-231 cells was blocked by the neutralizing antibody to IGF-IR but not TGF-β, FGF, and PDGF. Recombinant human IGF-I or IGF-II showed the greatest stimulation of anchorage-independent growth of the growth factors tested. The CM harvested from resorbed bone contained increased amounts of IGF-I compared with that harvested from control bone and IGF-I concentration was profoundly decreased in the CM harvested from bone in which bone resorption was inhibited by ZOL. Moreover, our in vivo studies showed that the number of mitosis was decreased in MDA/486STOP cells in which IGF-IR signaling was disrupted compared with that in MDA/EV. These results collectively suggest that IGF-I is at least one of the growth factors responsible for promotion of cancer cell colonization in bone.

Earlier clinical studies have reported that aggressive breast cancers express increased IGF-IR (14). However, it has not been examined whether human cancers spread to bone express elevated IGF-IR. Our immunohistochemical study using clinical specimens obtained at surgery exhibited that cancer cells colonizing bone were IGF-IR-positive regardless of the primary site of tumors. Although it is unknown whether these cancers also express IGF-IR at primary site, these observations suggest that IGF-IR expression is a requisite for metastatic cancer cells to appropriately respond to bone-derived IGFS to lead to bone metastases. Indeed, disruption of IGF signaling by introducing dominant-negative IGF-IR or short hairpin RNA against IGF-IR in MDA-MB-231 human breast cancer cells (MDA/486STOP or MDA/shGF-IR) significantly reduced bone metastases. Similarly, an anti-IGF neutralizing monoclonal antibody decreases tumor burden of MCF-7 human breast cancer in bone (30). Conversely, overexpression of wild-type IGF-IR in MDA-MB-231 cells (MDA/IGF-IR) significantly increased bone metastases in this study. Furthermore, we previously reported that the bone-seeking clone of MDA-MB-231 cells exhibits higher IGF-IR expression and responsiveness to IGF-I than parental cells (31). In contrast to these results, however, our in silico analysis of the published microarray database GSE14244 and GSE16554 that had been submitted to NCBI Gene Expression Omnibus.
survival factor that is activated by IGFs (12, 13, 21). We ing that activation of IGF-IR signaling by bone-derived IGFs the development and progression of bone metastases. PTHrP production and COX-2 and Jagged1 expression in breast IGFs that stimulate cell proliferation and TGF- promotes bone metastasis at least in part through releasing primary cytokines that play a critical role in the pathophysiology of bone metastasis of breast cancer (1–3). We have also reported that bone-derived TGF-β stimulates COX-2 expression in MDA-MB-231 cells and a COX-2 inhibitor reduces bone metastases (35). Moreover, it has recently been reported that bone-derived TGF-β increases Jagged1 expression in metastatic breast cancer cells, which directly stimulates osteoclast differentiation through activating Notch pathway (36). Taken together, our results suggest that osteoclastic bone resorption promotes bone metastasis at least in part through releasing IGFs that stimulate cell proliferation and TGF-β that increases PTHrP production and COX-2 and Jagged1 expression in breast cancer cells metastasized in bone, which consequently leads to the development and progression of bone metastases.

IGF has been also implicated in cell survival (12, 13). Our results showed that apoptosis in MDA/486STOP cells in bone was significantly increased compared with MDA/EV, suggesting that activation of IGF-IR signaling by bone-derived IGFs protected cancer cells from apoptosis. Akt is a well-described survival factor that is activated by IGFs (12, 13, 21). We confirmed that IGF-I activated Akt in MDA-MB-231 cells and found that this IGF-I–induced Akt activation was suppressed or enhanced in MDA/486STOP or MDA/IGF-IR cells, respectively. More importantly, suppression of Akt signals by introduction of dominant-negative Akt in MDA-MB-231 cells (MDA/DN-Akt) reduced bone metastases with increased numbers of apoptosis in cancer cells in bone. These results show that Akt is a downstream molecule of IGF/IGF-IR signaling and mediates antipapoptotic effects of IGFs on cancer cells in bone. Thus, IGF-induced Akt activation takes a part in the promotion of bone metastases.

NF-κB, another well-known survival factor (19), is one of the downstream signaling molecules of IGF/IR/Akt pathway (18, 20). Our in vitro studies showed that IGF-I activated NF-κB in MDA-MB-231 cells and the IGF-I–induced NF-κB activation was enhanced or suppressed in MDA/IGF-IR or MDA/486STOP, respectively. Our in vivo study showed that the dominant-negative inhibition of NF-κB by introducing the truncated IκBζ reduced bone metastases. Furthermore, NF-κB transcriptionally regulates the expression of a variety of cytokines including IL-1β, IL-6, TNFs, and macrophage colony-stimulating factor (M-CSF; ref. 19), all of which are stimulators of osteoclastogenesis and bone resorption (40). Activation of NF-κB may augment cancer-induced bone destruction through increased production of these osteoclastogenic cytokines. The result that osteoclast number was decreased in bone metastases of MDA/IkBζΔN cells supports this notion. It should be noted that the production of PTHrP was not changed in MDA/IkBζΔN cells compared with MDA/EV cells. It is therefore unlikely that the reduced number of osteoclasts in bone metastases of MDA/IkBζΔN cells is because of decreased PTHrP production and that IGF/IR/NF-κB pathway is involved in PTHrP-mediated osteoclastogenesis. In this context, Park and colleagues reported that granulocyte macrophage-colony stimulating factor is a target of NF-κB, which mediates osteolytic bone metastasis of breast cancer by stimulating osteoclast development (41). Contribution of NF-κB to osteoclast recruitment and bone resorption in the pathophysiology of bone metastasis remains to be elucidated.

IGFs are well-known mitogenic factors (12, 13). Our present study showed that IGFs increased colony formation of MDA-MB-231 cells. Furthermore, mitotic cells were decreased in MDA/486STOP colonized bone. Similar results were also observed in bone metastases of MDA/IkBζΔN but not in MDA/DN-Akt. These results suggest that IGF-IR signaling is not necessarily acting through Akt to activate NF-κB.

Despite that MDA/486STOP showed reduced bone metastases, tumor development of MDA/486STOP at the orthotopic mammary fat pad was not decreased compared with that of MDA/EV. Furthermore, pulmonary metastasis of MDA/486STOP or MDA/IGF-IR was changed to a marginal extent. These results raise the possibility that IGF actions are relatively bone-selective. Murphy and colleagues reported that the expression of IGF-I and IGF-II in lung and mammary gland is rarely detectable (42). Wu and colleagues have shown that
hepatic metastasis of colon cancer is reduced in the liverspecific IGF-I–deficient mice (43). From these earlier studies together with our results, it is possible that organ/tissue IGF concentration is influential on the behaviors of cancer cells arresting at the local site. In this regard, metastatic breast cancer cells with increased IGF-IR expression can take advantage of bone in which IGFs are abundantly stored. It is therefore expected that blockade of IGF/IGF-IR axis using agents including neutralizing monoclonal antibodies and small molecule tyrosine kinase inhibitors (12, 13) may suppress cancer cells in bone more effectively and selectively than other organs/tissues.

In conclusion, our data suggest that bone-derived IGFs, which are released from bone in substantial amounts by osteoclastic bone resorption, activate IGF-IR, Akt, and NF-xB signaling pathway, thereby increasing proliferation and decreasing apoptosis in breast cancer cells that are colonizing bone. These events consequently lead to the development and progression of bone metastases. Given the abundance of IGFs in bone, targeting IGF/IGF-IR/Akt/NF-xB signaling pathway may be a selective and effective approach for the treatment of bone metastases.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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