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Tumor-derived Platelet-derived Growth Factor-BB Plays a Critical Role in Osteosclerotic Bone Metastasis in an Animal Model of Human Breast Cancer[1]

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ABSTRACT

Breast cancer produces a variety of growth factors to promote its behavior at primary and secondary sites in autocrine/paracrine manners. However, the role of these growth factors in the colonization of cancer cells in bone, which is one of the most common metastatic sites, is poorly understood. To study this, we established an *in vivo* model in which the MCF-7 human breast cancer cells caused predominant osteosclerotic bone metastases 20–25 weeks after inoculation into the left cardiac ventricle in female nude mice. To make this model more time efficient, we overexpressed the oncogene *Neu,* which is associated with aggressive behavior in human breast cancers, in MCF-7 cells (MCF-7/Neu). MCF-7/Neu cells grew without estrogen and developed osteosclerotic bone metastases in 10–12 weeks in animals. Of note, MCF-7/Neu-bearing mice showed substantial plasma levels of human platelet-derived growth factor-BB (hPDGF-BB; 855 ± 347 pg/ml; mean ± SE, $n = 5$), indicating hPDGF-BB production by inoculated MCF-7/Neu cells. MCF-7/Neu cells in culture also produced large amounts of hPDGF-BB. Conditioned medium harvested from MCF-7/Neu cells stimulated osteoblastic bone formation in organ cultures of neonatal mouse calvariae, and a neutralizing antibody to hPDGF-BB blocked the osteoblastic bone formation. Stable transfection of the hPDGF-B AS in MCF-7/Neu cells reduced hPDGF-BB production in culture. Mice bearing these MCF-7/Neu cells with antisense showed reduced bone metastases with decreased plasma hPDGF-BB levels (54 ± 20 and 35 ± 21 in two different antisense and 696 ± 312 pg/ml in empty vector; mean ± SE; $n = 5$). Introduction of hPDGF-B cDNA in the MDA-MB-231 human breast cancer cells, which consistently formed osteolytic bone metastases, induced osteosclerotic lesions in the osteolytic bone metastases. In conclusion, we show that MCF-7 cells cause osteosclerotic bone metastases and that Neu enhances this capacity of MCF-7 cells. Our data suggest that MCF-7/Neu-derived hPDGF-BB plays a causative role in the development of osteosclerotic bone metastases in this model.

INTRODUCTION

Bone metastasis is one of the most common complications in patients with breast cancer. More than 70% of patients dying from breast cancer show skeletal involvement at autopsy (1). Identification of the molecules responsible for the preferential colonization of breast cancer cells in bone is, therefore, important for understanding the mechanism underlying bone metastases and useful for designing specific therapeutic interventions for bone metastases in breast cancer.

It has been widely recognized that breast cancer cells produce a variety of growth factors including IGF[3]-I and IGF-II, BMP, TGF-α, and PDGF in autocrine/paracrine manners to facilitate their growth and progression at primary and secondary sites (2, 3). Among these growth factors, PDGF has been shown to be produced in many human breast cancer cells (3, 4), and expression of PDGF B-chain in human breast carcinoma has been described (5). Moreover, human studies have reported that elevated levels of PDGF in the circulation and increased expression of PDGF in tumors correlate with increased metastases, lower response to chemotherapy, and shorter survival in breast cancer (6–8).

PDGF is a dimeric polypeptide growth factor comprised of AA, AB, and BB isoforms. It affects diverse biological events associated with tumorigenicity, invasion, and distant metastasis such as promotion of cell proliferation, cell migration, and angiogenesis (9, 10). Furthermore, PDGF, especially the BB isoform, is a well-known potent osteotropic factor that stimulates the functions of both osteoclasts and osteoblasts (11). It is, therefore, conceivable that PDGF-BB produced by breast cancers may exhibit significant influence on the development of bone metastases in which cancer cells, osteoclasts, and osteoblasts play key roles in the presence of complex interactions between them.

In the present study, we found that inoculation of the MCF-7 human breast cancer cells, which produce hPDGF-BB, into the left ventricle of the heart in female nude mice caused osteosclerotic bone metastases. Moreover, overexpression of the oncogene *Neu* into MCF-7 cells (MCF-7/Neu) increased hPDGF-BB production and the osteosclerotic bone metastases compared with MCF-7 cells. MCF-7/Neu tumor-bearing mice with bone metastases demonstrated elevated plasma levels of hPDGF-BB. Furthermore, inhibition of hPDGF-BB production in MCF-7/Neu cells by transfection of the PDGF-B AS significantly decreased osteosclerotic bone metastases. Our results suggest that hPDGF-BB produced in MCF-7/Neu cells is critical to the development of osteosclerotic bone metastases.

MATERIALS AND METHODS

Antibodies and Growth Factors. A polyclonal antibody to *Neu* (C-18) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A neutralizing polyclonal antibody to hPDGF-BB (AF-220-NA) and rhPDGF-BB, rhPDGF-AB, and rhPDGF-AA and rhBMP2 were purchased from R&D Systems (Minneapolis, MN).

Cells. The MCF-7 estrogen receptor-positive human breast cancer cells (12) were obtained from ATCC (Rockville, MD). The original MCF-7 cells required supplementation of estrogen (10^{-7} M) in the culture medium for their growth *in vitro*. Moreover, they also required s.c. implantation of estrogen pellets for intramammary tumor formation in normal female nude mice. In this study, however, we passaged these MCF-7 cells in phenol red-free DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS (Summit, Ft. Collins, CO), 2 mM L-glutamine, 0.11 g/l sodium pyruvate, 0.1% nonessential amino acid solution (Life Technologies, Inc., Grand Island, NY), 4.5 g/l glucose, 1% penicillin-streptomycin solution (Mediatech, Herndon, VA), and without estrogen supplementation to increase the sensitivity to exogenously added estrogen according to the method described by Masamura *et al.* (13).

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[3] The abbreviations used are: IGF, insulin-like growth factor; BMP, bone morphogenetic protein; TGF, transforming growth factor; PDGF, platelet-derived growth factor; rhPDGF-BB, recombinant human PDGF-BB; AS, antisense; ATCC, American Type Culture Collection; CM, conditioned medium; RT-PCR, reverse transcription-PCR; FBS, fetal bovine serum; EV, empty vector; PTH-rP, parathyroid hormone-related protein; ET, endothelin.

The FBS used here was not depleted with estrogen. Consequently, these MCF-7 cells acquired the capacity to proliferate in culture in the presence of relatively low levels of estrogen contained in FBS. However, these MCF-7 cells did not form intramammary tumor in the absence of s.c. implantation of estrogen pellets in normal female nude mice, demonstrating that their growth *in vivo* is dependent on estrogen (data not shown). We validated that these MCF-7 cells still retained the expression of functional estrogen receptor using Western blotting and estrogen responsive element reporter assay and responsiveness to estrogen in colony assay in soft agar (data not shown).

The MDA-231 human estrogen-independent breast cancer cells (14) were obtained from ATCC and cultured in DMEM supplemented with 10% FBS. All cells were routinely tested for *Mycoplasma* contamination.

Animal Model of Bone Metastasis. Bone metastasis was studied in a well-described animal model in which breast cancer cells were inoculated into the left ventricle of the heart in female nude mice as described previously (15–17). Subconfluent cells were fed with fresh medium 24 h before intracardiac injection. Cells (5×10^5 for MCF-7/Neu, 1×10^5 for MDA-MB-231) were suspended in 0.1 ml of PBS and injected into the left ventricle of 4–6-week-old female BALB/c-*nu/nu* mice (National Cancer Institute, Frederick, MD) with a 27-gauge needle under anesthesia with pentobarbital (0.05 mg/g). Estrogen was not supplemented in these experiments. Animals were kept in our animal facilities for 10–25 weeks as described (18).

Detection of Bone Metastases by Histology and Histomorphometry. Forelimb, hindlimb, and vertebral bones were removed from mice and fixed in 10% buffered formalin, decalcified in 14% EDTA for 2 weeks, and then embedded in paraffin. Sections were stained with H&E. Area of bone metastases and tumor burden were determined in middle sections under a microscope connected with Osteomeasure System (Osteometrics, Atlanta, GA).

Bone Formation in Organ Cultures of Neonatal Mouse Calvariae. The details of this assay have been described previously (19). Calvarial bones were excised from 4-day-old pups of Swiss white mice (Harlan, Indianapolis, IN), dissected free of adjacent connective tissues, placed in Biggers-Gwatokin-Jackson medium (Sigma Chemical Co.) containing 0.1% BSA, and incubated with 20% CM of breast cancer cells (v/v) in the absence or presence of an anti-hPDGF-BB antibody for 4 days. The Biggers-Gwatokin-Jackson medium medium has been widely and successfully used for organ cultures of bone (19). At the end of the incubation, bones were fixed in 10% buffered formalin overnight, decalcified in 14% EDTA for 4 h, and embedded in paraffin. Four-μm-thick sections were taken at a depth of 800 μm, lateral to the sagittal suture, and stained with H&E. Area of new bone and osteoblast number on the bone surfaces were determined by histomorphometry. rhBMP2 was used as a positive control.

Colony Formation Assay. Anchorage-independent growth was determined by colony formation in soft agar as described (20). Five hundred cells in 0.5 ml of 0.4% (w/v) agarose (Sea-plaque; FMC Corp., Rockland, ME) in DMEM were overlaid onto 0.5 ml of bottom layer of 0.6% agarose in 24-well culture plates and cultured for 7–14 days in a humidified CO_2 incubator at 37°C. At the end of the culture, colonies >100 μm in diameter were manually counted under an inverted microscope.

Measurement of hPDGF-BB. Blood from tumor-bearing mice was drawn by heart puncture at the time of sacrifice using disposal syringes with 27-gauge needles and transferred into blood collection tubes coated with EDTA (Vacutainer; Becton Dickinson, Franklin Lakes, NJ), and plasma was isolated. For measurement of hPDGF-BB production in MCF-7 cells in culture, CM was harvested from 48-h serum-free cultures. hPDGF-BB levels in these samples were measured using ELISA kits (R&D Systems) according to the manufacturer's instructions. The antibody used in this ELISA was shown to be specific for hPDGF-BB and does not cross-react with mouse PDGF-BB, according to information provided by the manufacturer.

Construction of hPDGF-B Chain Antisense Vector. A 570-bp *Bam*HI-*Bgl*II fragment of PSM-1 vector (ATCC), which contained the 5′ end of the *hPDGF B* gene and partial promoter region, was subcloned into the *Bam*HI site of pCDNA3.1zeo (+) (InVitrogen, Carlsbad, CA). Because of the compatibility of the sticky end of *Bam*HI and *Bgl*II, ligation occurred in both AS and sense orientation. The orientation of the subcloned gene was verified by size on the gel after digestion with a restriction enzyme *Pst*I. The sense vector was used as a control.

Transfection. Human Neu/HER-2 cDNA (kindly provided by Dr. Axel Ullrich, Germany) was subcloned into pCDNA3 vector carrying neomycin-resistant gene (InVitrogen) and transfected into MCF-7 cells using a Lipofectamine PLUS kit (Life Technologies, Inc.) in the presence of 1 mg/ml neomycin (InVitrogen). Stable clones (MCF-7/Neu cells) were established by limiting dilution and determined for expression of Neu by immunoblotting. In some experiments, the MCF-7/Neu cells were further transfected with the hPDGF-B chain AS construct using the same transfection procedures in the presence of 1 mg/ml zeocin (InVitrogen). MDA-231 cells were cotransfected with the hPDGF-B expression vector PSM-1 (ATCC) and pCDNA3 vector carrying neomycin-resistant gene (InVitrogen).

RT-PCR. Total RNA was extracted from cell cultures with the RNeasy Mini kit (Qiagen, Valencia, CA). RNA was used as a template for cDNA synthesis in a 20-μl volume containing 2.5 μM Oligo dT, 1 unit/μl Rnase inhibitor (Perkin-Elmer, Foster City, CA), 500 μM deoxynucleotide triphosphate mix (Life Technologies, Inc.), 10 mM DTT, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 200 units of SuperScipt II RNase H^- reverse transcriptase (Life Technologies, Inc.). The reaction was incubated at 42°C for 60 min. PCR analysis for AS expression was performed using primers that amplified a fragment of 234 bp. The 5′ primer was located on the vector, whereas the 3′ primer was in the antisense insert (5′ primer sequence, CACTGCTTACTGGCTTATC; 3′ primer sequence, AGTTGGACCTGAACATGA). No product was amplified if the insert was in the sense orientation. The PCR reaction mixture (final volume, 50 μl) contained 200 μM deoxynucleotide triphosphate mix, 1.5 mM $MgCl_2$, 2.5 units of Taq DNA polymerase (Life Technologies, Inc.), and 1 μl of cDNA template. A denaturation step of 95°C 5 min, followed by 32 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min, was used. The PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining under UV light. The RT-PCR for hPDGF receptor α, receptor β, and PTH-rP was performed as described before (21–23)

Statistical Analysis. All results are expressed as mean ± SE. Data were analyzed using two-sided Student's *t* test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Establishment of an Osteosclerotic Bone Metastasis Model. Inoculation of MCF-7 breast cancer cells into the left ventricle of the heart in female nude mice caused the formation of predominantly osteosclerotic bone metastases in leg bones 20–25 weeks after the inoculation without estrogen supplementation. The results obtained in several experiments showed that all mice (>30 mice) inoculated with MCF-7 cells had osteosclerotic bone metastases in their leg bones by 30 weeks. A representative radiograph showed the development of diffuse radiopaque lesions in the proximal tibia of MCF-7-bearing mice (Fig. 1, *left*). Histological examination of these lesions revealed sclerotic bones associated with metastatic MCF-7 breast cancer cells (Fig. 1, *right*). Thus, we established a rare animal model of breast cancer that reproducibly forms osteosclerotic bone metastasis. We then attempted to make this model more time and cost efficient for the subsequent experiments. In this attempt, we stably overexpressed the oncogene *Neu* into MCF-7 cells, which express marginal levels of endogenous Neu by Western analysis. Neu (or HER2 or ErbB-2) is a member of epidermal growth factor receptor tyrosine kinase family and shown to be overexpressed in >25% of breast cancers patients with poor prognosis (24), suggesting that Neu increases aggressiveness in breast cancer behavior. Consistent with this notion, it has been reported that MCF-7 cells stably transfected with Neu acquire the capacity to grow in an estrogen-independent manner *in vitro* and *in vivo* (25). Indeed, our MCF-7/Neu cells also grew without estrogen supplementation beyond that contained in normal FBS *in vitro* and formed intramammary tumors in the absence of estrogen pellets in normal female nude mice (data not shown). Mice inoculated with these MCF-7/Neu cells showed accelerated development of osteosclerotic bone metastases with identical radiological and histological characteristics to those of MCF-7 cells. More than 85% of animals inoculated with MCF-7/Neu cells developed discernible osteosclerotic

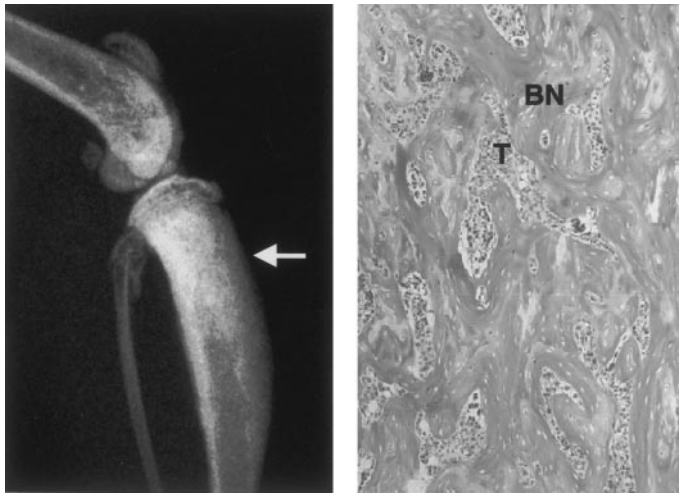

Fig. 1. Development of osteosclerotic bone metastasis after intracardiac inoculation of MCF-7 breast cancer cells. *Left*, a representative radiograph shows the formation of demarcated radiodense lesions in the proximal tibia 25 weeks after the inoculation (*arrow*). *Right*, histological examination revealed new bone formation (*BN*) associated with metastatic MCF-7 breast cancer cells (*T*) in these lesions. MCF-7/Neu cells developed the identical lesions as early as 10–12 weeks after inoculation. H&E staining, ×100.

bone metastases 10–12 weeks after the inoculation. Accordingly, we used MCF-7/Neu cells in the following *in vivo* experiments.

It should be noted that extensive histological examination revealed that there were small osteolytic lesions associated with the presence of osteoclasts in the osteosclerotic bone metastases of MCF-7 and MCF-7/Neu tumor (data not shown).

Plasma hPDGF-BB Levels in Mice with MCF-7/Neu Bone Metastases. Several groups including ours have reported previously that PTH-rP plays a central role in osteolytic bone metastases in breast cancer in human and animals (23, 26–28). Because MCF-7/Neu caused osteosclerotic bone metastases and PTH-rP is a potent stimulator of osteoclastic bone resorption, it is unlikely that PTH-rP plays a role in this model. In addition, in contrast to the previous reports (29), MCF-7/Neu cells we used in this study did not express and produce PTH-rP, as determined by RT-PCR and immunoradiometric assay (data not shown). Accordingly, we searched for other osteotropic factors that were shown to be produced in MCF-7 cells and focused on PDGF-BB. MCF-7 cells are shown to produce PDGF (3, 4), and PDGF is a potent stimulator of bone formation (11). Furthermore, clinical studies reported that elevated serum levels of PDGF and increased expression of PDGF in tumors correlate with increased metastases in breast cancer patients (6–8).

We measured plasma levels of hPDGF-BB in non-tumor-bearing mice and MCF-7/Neu-bearing mice with bone metastases by ELISA at the time of sacrifice (12 weeks after cell inoculation). MCF-7/Neu-bearing mice with bone metastases demonstrated elevated plasma levels of hPDGF-BB (855 ± 347 pg/ml; mean ± SE; $n = 5$) compared with non-tumor-bearing animals (undetectable). This result suggests that MCF-7/Neu cells produce and secrete considerable amounts of hPDGF-BB into the circulation in MCF-7/Neu tumor-bearing mice. Because the antibody used in this ELISA specifically recognizes hPDGF, the PDGF detected in plasma of these tumor-bearing mice is of human origin and thus derived from MCF-7/Neu cells.

hPDGF-BB Production in MCF-7 Breast Cancer Cells *in Vitro*. To verify that elevated circulating hPDGF-BB is derived from MCF-7/Neu cells, we next determined the production of hPDGF-BB in MCF-7/Neu cells in culture. ELISA showed that CM harvested from MCF-7/Neu cell cultures contained substantial amounts of hPDGF-BB (Fig. 2). hPDGF production was much greater in MCF-7/Neu cells than MCF-7 cells (Fig. 2). In contrast, CM harvested from the MDA-231 human breast cancer cells that developed osteolytic bone metastases (Refs. 15–17; see also Fig. 7, *left*) exhibited undetectable hPDGF-BB production (Fig. 2).

To determine whether hPDGF-BB produced in MCF-7/Neu cells is an autocrine factor for MCF-7/Neu cells, expression of hPDGF receptor α and β in MCF-7/Neu cells was examined by RT-PCR. We found that there was no expression of hPDGF receptor α and β in MCF-7/Neu cells (data not shown), suggesting that hPDGF-BB produced in MCF-7/Neu cells does not function as an autocrine factor but rather a paracrine factor.

Effects of CM Harvested from the MCF-7 Cell Cultures on Bone Formation in Neonatal Mouse Calvariae in Organ Culture. Our findings that MCF-7/Neu breast cancer cells developed osteosclerotic bone metastases with substantial plasma levels of hPDGF-BB in tumor-bearing animals and produced large amounts of hPDGF-BB in culture together with the fact that PDGF-BB is a potent stimulator of bone formation (11) suggest that hPDGF-BB produced in MCF-7 cells stimulate osteosclerosis in bone metastases in MCF-7/Neu breast cancer. To test this notion, we examined whether CM harvested from MCF-7/Neu breast cancer cell cultures promoted bone formation in neonatal mouse calvariae in organ cultures. In a preliminary experiment, we found that the MCF-7/Neu CM at concentrations of 5, 10, and 20% (v/v) promoted bone formation in a dose-dependent manner in this organ culture assay (data not shown), and accordingly, 20% CM was tested in the following experiments. Histological examination revealed that the CM of MCF-7/Neu cell cultures stimulated new bone formation with increased numbers of osteoblasts on the periosteal surfaces of old bones to an equivalent extent to rhBMP2 (50 ng/ml; Fig. 3*A*; data not shown for BMP2). CM of MCF-7 showed less activity of new bone formation and stimulation of osteoblast number than the MCF-7/Neu CM (Fig. 3*A*). Histomorphometric analysis also showed that the CM of MCF-7/Neu more potently stimulated new bone formation (Fig. 3*B*, *left*) and osteoblast number (Fig. 3*B*, *right*) than MCF-7 CM.

Effects of a Neutralizing Antibody to hPDGF-BB on Bone Formation in Mouse Neonatal Calvariae in Organ Culture. To examine whether hPDGF-BB is responsible for the bone formation-stimulating activity in the MCF-7/Neu CM, we next tested the effects of a neutralizing antibody specific to hPDGF-BB. The antibody (1 μg/ml) partially but significantly inhibited the bone formation-stimulating activity of the MCF-7/Neu CM (Fig. 4, *left*). However, the antibody at a concentration of 1 (Fig. 4, *left*) or 10 μg/ml (data not shown) failed to completely suppress the bone formation-stimulating

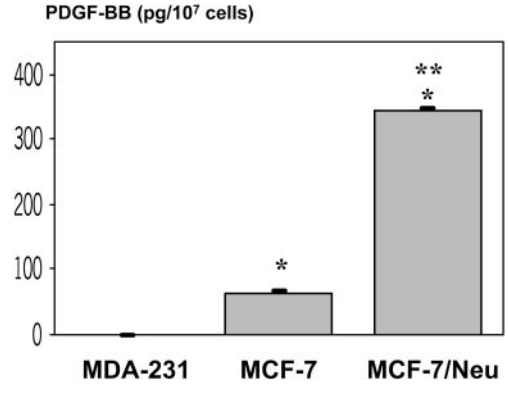

Fig. 2. hPDGF-BB production in MCF-7 and MDA-231 breast cancer cells in culture. Serum-free 48-h culture supernatants were harvested. hPDGF-BB levels were measured by ELISA. Data are shown as means; *bars*, SE. *, significantly greater than MDA-231 ($n = 5$; $P < 0.001$). **, significantly greater than MCF-7 ($n = 5$; $P < 0.001$).

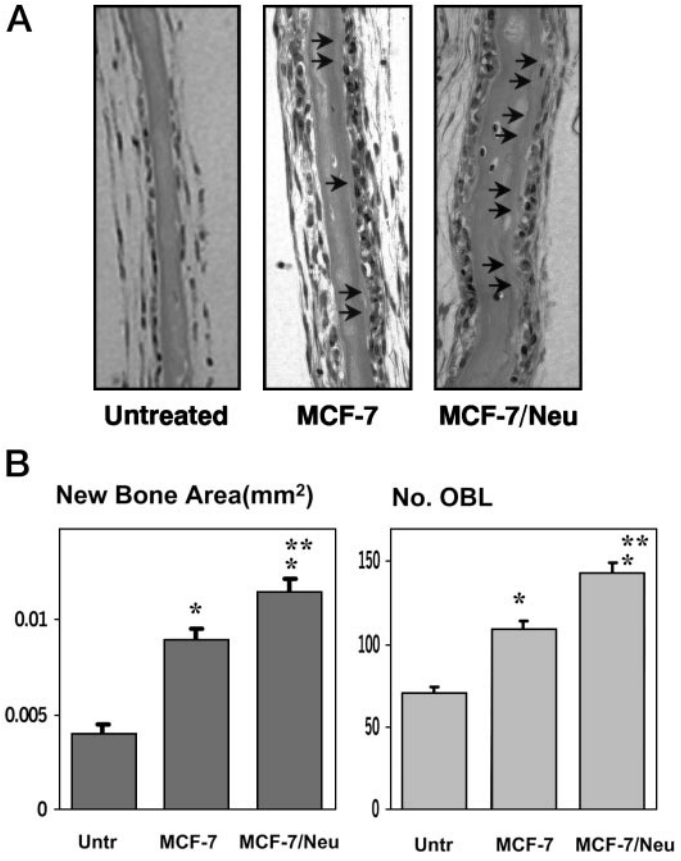

Fig. 3. Histological (*A*) and histomophometrical (*B*) examination of the effects of MCF-7 and MCF-7/Neu CM on bone formation in neonatal mouse calvariae in organ culture. Serum-free CM was harvested from confluent cultures and tested for bone-forming activity using the neonatal mouse calvarial assay (20% CM plus 80% fresh media, v/v). Bones were cultured for 4 days and then processed for histological examination. *A*, new bone formation was observed on the top of old bone (*arrows*) with numerous active osteoblasts on the surface of new bone (H&E, ×400). *B*, new bone area (*left*) and osteoblast number (*right*) were measured by histomorphometry as described in the text. Data are shown as means; *bars*, SE. ∗, significantly greater than untreated bones ($n = 4$; $P < 0.001$). ∗∗, significantly greater than MCF-7 CM-treated bones ($n = 4$; $P < 0.05$).

activity, suggesting the presence of an additional bone formation-stimulating activity to hPDGF-BB in the MCF-7/Neu CM. Consistent with this notion, the same neutralizing antibody failed to inhibit the bone formation-stimulating activity of the MCF-7 CM (Fig. 4, *right*), again suggesting that a factor(s) other than hPDGF-BB is contained in the MCF-7 CM.

We confirmed that this antibody at 1 and 10 μg/ml had no effects on bone formation and morphology of osteoblasts in untreated calvariae (data not shown), suggesting that the inhibition by the antibody is unlikely attributable to cytotoxicity. Control IgG had no effects. These results suggest that hPDGF-BB produced in MCF-7/Neu cells is, in part, responsible for the stimulation of bone formation in mouse calvarial bones in organ culture. Consistent with this notion, rhPDGF-BB (50 ng/ml) markedly stimulated new bone formation, whereas the same concentration of PDGF-AB or PDGF-AA isoform showed marginal or no stimulation of new bone formation, respectively (data not shown).

Inhibition of Osteosclerotic Bone Metastases of MCF-7/Neu Breast Cancer by hPDGF-B AS cDNA. Our *in vitro* results suggest that hPDGF-BB produced in MCF-7/Neu breast cancer cells contributes to the development of osteosclerotic bone metastases. To test this notion *in vivo*, we examined the effects of inhibition of hPDGF-BB production in MCF-7/Neu breast cancer by stable transfection of an AS construct of hPDGF-B chain on the development of osteosclerotic bone metastases. Stable AS hPDGF-B transfectants were screened for hPDGF-BB production using ELISA. We selected two clones (MCF-7/Neu/PDGFB.AS.5 and MCF-7/Neu/PDGFB.AS.8), which showed the expression of the AS hPDGF-B message by RT-PCR (data not shown) and markedly reduced hPDGF-BB production (Fig. 5). We subsequently inoculated them into the left ventricle of the heart in female nude mice. Histological (Fig. 6*A*) and histomorphometrical (Fig. 6*B*) examination demonstrated that bone metastases in MCF-7/Neu/PDGFB.AS.5 and MCF-7/Neu/PDGFB.AS.8 were markedly decreased compared with EV-transfected control MCF-7/Neu cells. Moreover, tumor-induced osteosclerosis was also decreased (Fig. 6*C*). In conjunction with these findings, plasma levels of hPDGF-BB were also significantly decreased in mice bearing MCF-7/Neu/PDGF-B.AS.5 and MCF-7/Neu/PDGFB.AS.8 tumor (54 ± 20 in AS.5, 35 ± 21 in AS.8, and 696 ± 312 pg/ml in EV).

Effects of hPDGF-B cDNA Introduction on Osteolytic Bone Metastases in the MDA-231 Human Breast Cancer. We have reported previously (15–17) that the MDA-231 human breast cancer cells cause osteolytic bone metastases associated with osteoclastic bone destruction after intracardiac inoculation in female nude mice

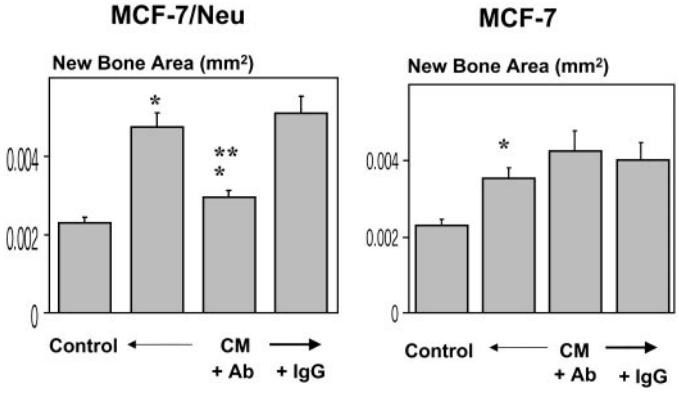

Fig. 4. Histomophometrical examination of the effects of a neutralizing antibody to hPDGF-BB on new bone formation induced by MCF-7/Neu CM (*left*) and MCF-7 CM (*right*). Neonatal mouse calvariae were treated with MCF-7/Neu CM or MCF-7 CM (20%) in the absence or presence of the antibody (1 and 10 μg/ml). New bone formation and osteoblast number were measured as described in the text. Both parameters showed the identical results, and here only the data of new bone area are shown. Data for 10 μg/ml are not shown. Data are shown as means; *bars*, SE. ∗, significantly greater than untreated bones ($n = 4$; $P < 0.01$). ∗∗, significantly smaller than MCF-7 CM-treated bones ($n = 4$; $P < 0.05$).

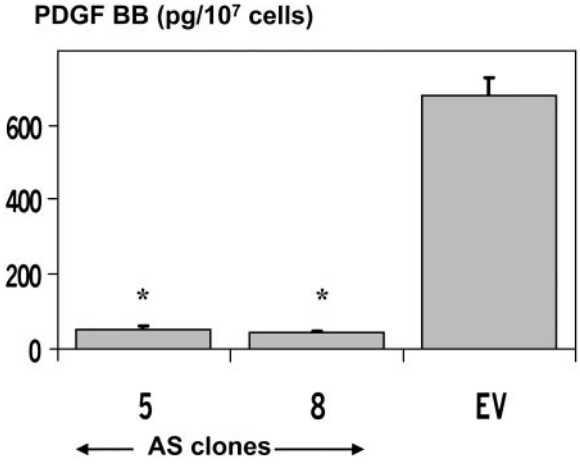

Fig. 5. hPDGF-BB production in MCF-7/Neu cells transfected with PDGF-B AS construct. hPDGF-BB levels in serum-free culture supernatants were measured by ELISA. Data are shown as means; *bars*, SE. ∗, significantly less than EV-transfected cells ($n = 3$; $P < 0.001$).

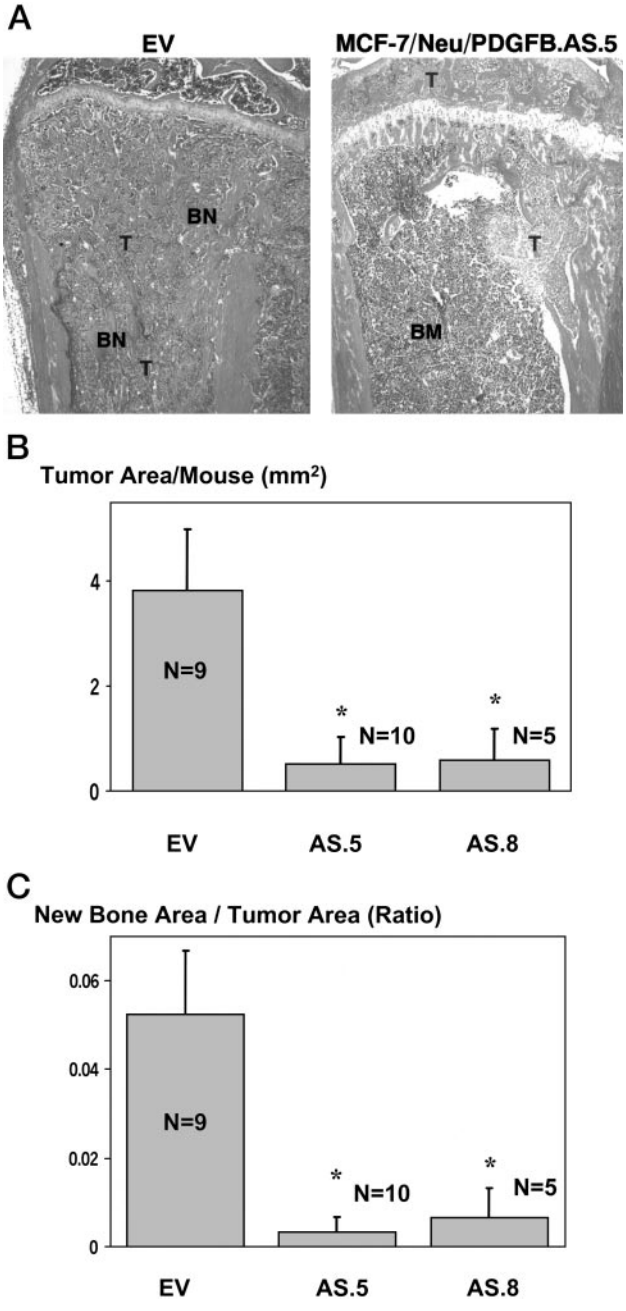

Fig. 6. Decreased osteosclerotic bone metastases in MCF-7/Neu cells transfected with PDGF-B AS constructs or EV. MCF-7/Neu/PDGFB.AS.5, MCF-7/Neu/PDGFB.AS.8, or MCF-7/Neu.EV cells were inoculated into the left ventricle of female nude mice as described. Bones were harvested and processed for histology 10 weeks after cell inoculation. *A*, EV-transfected MCF-7/Neu (*left*). MCF-7/Neu tumor (*T*) completely occupies the bone marrow cavity. There is profound tumor-induced new bone formation (*BN*). *Right*, MCF-7/Neu/PDGFB, AS.5. The tumor burden (*T*) of MCF-7/Neu/PDGFB, AS.5 is markedly reduced compared with MCF-7/Neu. There is little new bone formation associated with tumor. *BM*, normal bone marrow (H&E, ×40). *B*, area of MCF-7/Neu/PDGFB.AS.5, MCF-7/Neu/PDGFB.AS.8, or MCF-7/Neu.EV tumor in bone was measured by histomorphometry. Data are shown as means; *bars*, SE. ∗, significantly less than the EV group ($P < 0.05$). *C*, new bone formation associated with MCF-7/Neu/PDGFB.AS.5, MCF-7/Neu/PDGFB.AS.8, or MCF-7/Neu.EV tumor as measured by histomorphometry. Data are shown as means; *bars*, SE. ∗, significantly less than the EV group ($P < 0.05$).

(Fig. 7, *left*). We have shown here that MDA-231 cells produce undetectable levels of hPDGF-BB, whereas MCF-7 and MCF-7/Neu cells produce hPDGF-BB and develop osteosclerotic bone metastases (Fig. 2). Collectively, these results suggest that hPDGF-BB production influences the histological pattern of bone metastases in breast cancer. Accordingly, we tested a hypothesis that introduction of hPDGF-B cDNA may change the histological pattern of bone metastasis of MDA-231 breast cancer from osteolytic to osteosclerotic. In support of this hypothesis, MDA-231 cancer stably transfected with hPDGF-B cDNA (MDA-231/PDGF-B) developed the mixed type bone metastases containing osteosclerotic lesions in some parts of osteolytic lesions (Fig. 7, *right*). We found that PTH-rP production, which plays a central role in the development of osteolytic bone metastases in MDA-231 cancer (27, 28), was not different between MDA-231/PDGF-B and nontransfected MDA-231 (data not shown).

DISCUSSION

Bone cell activity *in vivo* is normally balanced under the control of local cytokines and growth factors (30). Once cancer metastasizes to bone, this balance is disrupted because of elevated local levels of autocrine/paracrine growth factors overproduced by metastatic cancer cells. This may in turn cause excessive osteoclastic bone resorption or osteoblastic bone formation, leading to the development of osteolytic or osteosclerotic bone metastases, respectively. In the present study, we have shown that the MCF-7/Neu human breast cancer cells, which develop osteosclerotic bone metastases *in vivo*, produce considerable amounts of hPDGF-BB, thereby elevating circulating levels of hPDGF-BB in MCF-7/Neu tumor-bearing animals. Inhibition of hPDGF-BB production by transfection of the hPDGF-B AS markedly reduced osteosclerotic bone metastases with decreased circulating hPDGF-BB levels in tumor-bearing animals. Consistent with these *in vivo* results, CM of MCF-7/Neu cells, which contains large amounts of hPDGF-BB, stimulated bone formation with increased numbers of osteoblasts in organ cultures of neonatal mouse calvariae. A neutralizing antibody specific for hPDGF-BB inhibited the stimulatory effect of the MCF-7/Neu CM. Furthermore, rhPDGF-BB and rhPDGF-AB but not rhPDGF-AA stimulated bone formation in the same organ culture assay. Finally, overexpression of hPDGF-B chain cDNA in the MDA-231 human breast tumor, which showed undetectable levels of hPDGF-BB production in culture and consistently caused osteolytic bone metastases (15–17), induced the development of osteosclerotic lesions in some parts of its osteolytic bone metastases. Taken together, these results strongly suggest that hPDGF-BB produced in MCF-7/

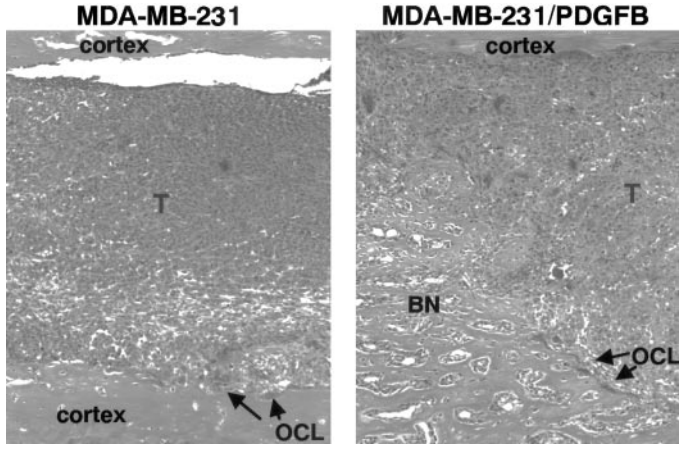

Fig. 7. Effect of PDGF-B chain cDNA overexpression on the development of bone metastases in MDA-231 cells. MDA-231 cells overexpressing PDGF-B chain cDNA were inoculated into the left cardiac ventricle of female nude mice as described. Mice were sacrificed 4 weeks after cell inoculation, and bones were subjected to histological examination. Nontransfected MDA-231 cells show osteolytic lesions with no osteosclerotic lesions (*left*), whereas PDGFB-overexpressing cells form the mixed type bone metastases containing osteosclerotic lesions (*BN*) in some parts of osteolytic lesions (*right*). *Arrows*, active osteoclastic bone resorption. There is also active osteoclastic bone resorption along the endosteal surfaces. H&E, ×100.

Neu cells plays a critical role in the development of osteosclerotic bone metastases in this animal model.

The effects of Neu overexpression on MCF-7 cells are of note. MCF-7/Neu cells show the capacity to develop osteosclerotic bone metastases significantly faster than MCF-7 cells when inoculated in female nude mice. Our findings that: (*a*) MCF-7/Neu cells produce increased amounts of hPDGF-BB compared with MCF-7 cells; (*b*), a neutralizing antibody specific for hPDGF-BB inhibits bone formation-stimulating activity of MCF-7/Neu CM but not MCF-7 CM in mouse neonatal calvarial assay; and (*c*) PDGF-B AS profoundly decreased osteosclerotic bone metastases in MCF-7/Neu-bearing mice collectively suggest that increased hPDGF-BB production attributable to Neu overexpression is responsible for accelerated development of osteosclerotic bone metastases. In addition to increased PDGF production, MCF-7/Neu cells also exhibit the capacity to grow in the presence of relatively low levels of estrogen *in vitro* and *in vivo*, demonstrating that Neu confers growth aggressiveness on MCF-7 cells. It is, thus, probable that increased capacity of growth is also one of the mechanisms underlying accelerated development of osteosclerotic bone metastases in MCF-7/Neu-bearing mice. Finally, it should be noted that our recent preliminary experiment shows increased BMP-2 mRNA expression in MCF-7/Neu cells compared with MCF-7 cells (data not shown), suggesting an involvement of BMP-2 in enhanced osteosclerotic bone metastases.

The observation that a specific neutralizing antibody for hPDGF-BB inhibited the bone formation-stimulating activity of MCF-7/Neu CM but not MCF-7 CM together with the result that MCF-7 cells produce marginal amounts of hPDGF-BB suggests that the osteosclerotic bone metastases caused by MCF-7 cells are most unlikely attributable to hPDGF-BB. It is currently unknown by what mechanism MCF-7 cells induce osteosclerotic bone metastases. It has been reported that MCF-7 cells produce a variety of growth factors such as TGF-β, IGFs, BMPs, fibroblast growth factors, ET-1 (3, 31, 32), all of which are potent osteotropic factors. In preliminary experiments, we indeed observed that our MCF-7 cells expressed BMP-2, TGF-β, fibroblast growth factor-2, and ET-1 by RT-PCR and showed the production of several peaks of bone formation-stimulating activity after fractionation on a heparin-Sepharose affinity column chromatography (data not shown). Moreover, our group has demonstrated recently that ET-1 causes osteosclerotic bone metastases in MCF-7 and ZR-75-1 human breast cancer using the same animal model (33). Taken together, it is suggested that factors other than hPDGF-BB are responsible for the osteosclerosis in the bone metastases caused by MCF-7 cells.

Because our data show that there is no expression of PDGF receptors in MCF-7/Neu cells, hPDGF-BB produced in MCF-7/Neu cells functions as a paracrine factor for neighboring cells during the development of osteosclerotic bone metastases. Because osteoblasts are shown to have PDGF receptors (11), they are probably one of the target cells of MCF-7/Neu-derived hPDGF-BB and stimulated to form excess bone in response to hPDGF-BB, leading to the development of predominantly osteosclerotic bone metastases. Moreover, because PDGF is a well-recognized stimulator of angiogenesis, other likely targets of MCF-7/Neu-derived hPDGF-BB are bone marrow endothelial cells (9, 10), which play an important role in tumor-associated angiogenesis in bone. Thus, hPDGF-BB produced by MCF-7/Neu cells may cause diverse biological events associated with bone formation in a paracrine manner, which in turn may cumulatively promote the progression of osteosclerotic bone metastases.

Although we did not observe radiologically prominent osteolytic lesions in the bone metastases of MCF-7/Neu breast cancer, extensive histological examination revealed the occurrence of active osteoclastic bone resorption in some parts in the MCF-7/Neu osteosclerotic bone metastases. This observation suggests that osteoclasts as well as osteoblasts are influenced by MCF-7/Neu-derived PDGF-BB. Studies from several groups including our group have demonstrated that PTH-rP produced by breast cancer cells play a causative role in the development of osteolytic bone metastases in breast cancer (23, 26–28). However, the MCF-7/Neu cells we used in this study do not express PTH-rP mRNA. It, therefore, seems likely that an additional tumor-derived factor or hPDGF-BB is responsible for the osteolysis observed in the MCF-7/Neu bone metastases. In this context, osteoclasts are shown to possess PDGF receptors (34), and PDGF-BB has been shown to be a potent stimulator of osteoclastogenesis and bone resorption (11, 34). Whether hPDGF-BB produced in MCF-7/Neu cells plays a role in the osteolysis is unknown.

Osteosclerotic bone metastases are most common in prostate cancer, and most cases of bone metastases in breast cancer ($>$60%) are osteolytic (35), raising an argument that the MCF-7/Neu bone metastasis model described here is not relevant. However, it has been recognized that some breast cancers ($\approx$10%) manifest predominant osteosclerotic bone metastases (35, 36), suggesting that osteosclerotic bone metastases in breast cancer are not uncommon. Although Nemeth *et al.* (37) have reported recently a unique model of osteoblastic bone metastasis of prostate cancer, there is still a very limited number of animal models of prostate cancer that reproducibly form osteosclerotic bone metastases. It is, therefore, suggested that pieces of information obtained using the MCF-7/Neu model might be helpful for elucidating the mechanism of osteosclerotic bone metastases in prostate cancers.

In conclusion, our results suggest that the MCF-7/Neu breast cancer develops osteosclerotic bone metastases through the production of hPDGF-BB in a paracrine fashion. Extensive clinical studies are necessary to determine whether our experimental findings described here can be extrapolated to osteosclerotic bone metastases in breast cancer patients.

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Tumor-derived Platelet-derived Growth Factor-BB Plays a Critical Role in Osteosclerotic Bone Metastasis in an Animal Model of Human Breast Cancer

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