Parathyroid Hormone-related Peptide Regulates Matrix Metalloproteinase-13 Gene Expression in Bone Metastatic Breast Cancer Cells

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Abstract. Background: Breast cancer (BC) cells often metastasize to bone where they express large amounts of parathyroid hormone-related protein (PTHrP). In this study, we investigated the possibility that PTHrP may have roles in breast cancer bone metastasis independently of, or in addition to, its roles in osteoclastic function. Materials and Methods: A mouse model of bone metastasis was prepared by inoculating mice with suspensions of the human BC cell line MDA-MB-231 tumor cells via the left cardiac ventricle. Matrix metalloproteinase-13 (MMP-13) expression in the bone microenvironment was examined by Western blot and Real-time RT-PCR (RT-PCR) analysis, as well as by confocal microscopy. Results: The invading MDA-MB-231 cells contained conspicuous amounts of both PTHrP and MMP-13, an important matrix-degrading enzyme; and treatment of the cells in culture with exogenous PTHrP markedly stimulated MMP13 gene expression. Analysis of signaling mechanisms showed that PTHrP treatment led to rapid increases in the levels of phosphorylated protein kinase C (PKCα) and extracellular signal-regulated kinase (ERK1/2). Pharmacologic inhibition of ERK1/2 and PKC as well as Pf PKA activities counteracted the PTHrP-dependent stimulation of MMP13 expression. Indeed, pharmacologic activation of PKA or PKC was sufficient for stimulation of MMP13 expression. Conclusion: Consistent with these findings, the inhibition of PKC prevented PTHrP-induced activation of ERK1/2, whereas 12-O-tetradecanoylphorbol-13-acetate (TPA), a stimulator of PKC, up-regulated the PTHrP-induced activation of ERK1/2. Taken together, our data indicate that the MDA-MB-231 breast cancer cells may carry out bone destruction and favor their own metastatic behavior by producing MMP-13. Given that the cells expressed PTHrP and that this factor stimulated MMP-13 expression, metastatic bone destruction may result from a PTHrP autocrine loop involving a PKC-ERK1/2 signaling pathway.

Bone metastasis of breast cancer is predominantly osteolytic and also causes skeletal lesions including pathologic fracture, intractable bone pain, nerve compression, and hypercalcemia (1, 2). Parathyroid hormone-related protein (PTHrP) is a paracrine factor that was initially identified for its involvement in hypercalcemia associated with malignancy (3-5). Clinical and experimental evidence, however, provided evidence for an additional and potentially important role for PTHrP in malignancy, namely, its function as a mediator of bone destruction during osteolytic metastasis. Neutralizing antibodies against PTHrP inhibit both the development and progression of bone metastasis by cells of the human breast cancer cell line MDA-MB-231 in a mouse model (6, 7). PTHrP produced by the breast cancer cells enhances osteoclastic activity via the expression of osteoblastic receptor activator of nuclear factor kappa B ligand (RANKL) and vascular endothelial growth factor (VEGF) (8, 9). Thus, PTHrP expression by breast cancer cells may provide a selective growth and invasive advantage within the bone microenvironment by its apparent ability to stimulate osteoclastic activity and bone resorption.

Matrix metalloproteinase-13 (MMP-13) was originally identified in breast carcinoma cells overexpressing it (10), and is also produced by a variety of malignant tumors (11, 12). MMP-13 can degrade type I, II, III and IV collagens, cartilage...
proteoglycan aggrecan, and other extracellular matrix components (13); and it plays roles in tumor-mediated bone resorption (14). Furthermore, results from in vitro and in vivo studies indicate that MMP-13 is strongly induced in osteoblastic cells and femora by PTH (15, 16). However, there are only limited data concerning the topographic expression patterns of MMP-13 within metastatic bone tumors that could provide insights into its role in bone metastasis (17). There is also limited knowledge as to how MMP-13 may be regulated in bone metastatic tumor cells. The present study was conducted to address these important questions.

**Materials and Methods**

**Materials.** Human PTHrP(1-34), dibutyryl-cAMP (db-cAMP), and 12-O-tetradecanoylphorbol-13-acetate (TPA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). P300 were from Calbiochem (La Jolla, CA, USA); and anti-PTHrP, PTH1R, PKCα, p-PKCα, ERK1/2, and p-ERK1/2 antibodies, from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-MMP-13 antibodies were purchased from Neo Markers (Fremont, CA, USA).

**Cell culture.** A highly metastatic variant of MDA-MB-231 cells (18, 19) was cultured in Dulbecco’s modified Eagles medium (DMEM; Invitrogen Corporation, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS, USA) and 1% penicillin/streptomycin (Invitrogen Corporation) in an atmosphere of 5% CO2/air at 37˚C.

**Intracardiac injection of MDA-MB-231 cells in nude mice.** A mouse model of bone metastasis was prepared by inoculating 5-week-old female BALB/C nude mice (CLEA JAPAN, INC, Tokyo, JAPAN) with tumor cell suspensions of MDA-MB-231 cells (10^5 cells/100 μl of phosphate-buffered saline (PBS)) via the left cardiac ventricle, as described previously (19, 20). On day 25, radiographs were obtained and the hind limbs were processed as described in the following subsections. The Animal Committee of Okayama University Graduate School of Medicine and Dentistry approved all of the experimental procedures.

**Immunohistochemical analysis.** Hind limbs were fixed with 10% neutral phosphate-buffered formalin, decalcified in a 14% EDTA solution for 2-3 weeks, and embedded in paraffin. Sections were cut at a thickness of 5 μm and treated with xylene and ethanol to remove the paraffin. Antigen retrieval by microwave treatment for 15 min in a 500 W microwave oven was performed in Target Retrieval Solution (DAKO, Carpinteria, CA, USA). A blocking step with FBS was performed. The first antibody in 3% bovine serum albumin (BSA)-PBS was applied overnight in a moist chamber at 4˚C. The sections were then washed 3 times with PBS and reacted with the second antibody in 3% BSA-PBS for 1 h. They were finally washed 3 times with PBS, and the coverslips were mounted on glass slides and viewed under a confocal microscope (Bio-Rad, Laboratories, Hercules, CA, USA).

**Immunoprecipitation.** MDA-MB-231 cells were grown to confluence in 100-mm diameter dishes and then treated with increasing concentrations of PTHrP. The culture medium was collected 24 h after addition of PTHrP and centrifuged for 5 min at 4˚C. Aliquots (1 μl) of the supernatant were then mixed with 20 μl of uncoated protein G-Agarose beads (Santa Cruz Biotechnology) for 30 min at 4˚C. The beads were pelleted by centrifugation at 2500 rpm for 5 min at 4˚C, and the resulting cleared supernatants were transferred to fresh 1.5 ml microcentrifuge tubes at 4˚C and mixed with 2 μl of MMP-13 antibodies (approximately 2 μg). After overnight incubation at 4˚C, 20 μl of proteinG-Agarose beads were added and the mixtures were incubated overnight at 4˚C on a rotator. The beads were recovered by centrifugation, rinsed 3 times with lysis buffer, resuspended in sample buffer (50 mM Tris-HCl, pH 6.8, containing 2% sodium dodecyl sulfate (SDS) 0.1% bromophenol, and 10% glycerol) and boiled. Proteins were size-separated on SDS-polyacrylamide gels and transferred to nylon membranes (Immobilon-P, Millipore Co., Bedford, MA, USA).

**Real-time RT-PCR.** Total RNA was isolated by using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. Complementary DNA was generated from 1 μg of total RNA in a final volume of 20 μl by using a First Strand cDNA Synthesis Kit (Invitrogen). Real-time RT-PCR was performed with a Light Cycler (Roche Molecular Biochemicals, Mannheim, Germany) in Light Cycler capillaries by using a commercially available master mix containing Taq DNA polymerase and SYBR-Green I (Roche Molecular Biochemicals). After the addition of primers, MgCl2 (3 mM), and template DNA to the master mix, 65 cycles of denaturation (95˚C for 10 s), annealing, (55˚C for 5 s), and extension (72˚C for 10 s) were performed. The primers used were the following: 5’-TAACCGTATTGTTCGCGTCA-3’ and 5’-TCCAGCCACCTGTTGCTGTA-3’ for GAPDH, 5’-TGAACGGGAAGTCACTGG-3’ and 5’-TCCACCCCTTGTGGTCCTA-3’ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). After the completion of PCR amplification, a melting curve analysis was performed. The fluorescence intensity of the double strand-specific SYBR-Green I, reflecting the amount of formed PCR product, was monitored at the end of each elongation step. GAPDH mRNA levels were used to normalize the cDNA content of the samples.

**Immunofluorescence analysis.** After growth on culture slides, MDA-MB-231 cells were washed with PBS, fixed for 20 min with cold methanol, and permeabilized with 0.1% NP-40 in PBS. After washing 3 times with PBS, the cells were incubated for 1 h with first antibodies in 3% BSA-PBS, washed again 3 times with PBS, and reacted with second antibodies in 3% BSA-PBS for 1 h. After a final wash, the cells were sealed with a coverslip and viewed under a confocal microscope (Bio-Rad Laboratories).
Subcellular fractionation. Subcellular fractionation of control and treated MDA-MB-231 cells into cytosol and membrane fractions was performed as follows: Monolayer cultures were washed 3 times with ice-cold PBS buffer and scraped into cold homogenization buffer (HB) containing 20 mM Tris-HCl (pH 7.4), 4 mM EDTA, 2 mM EGTA, 10% glycerol, 1 μg/ml leupeptin, and 1 mM PMSF. The cells were lysed via sonication 4 times at 15-s intervals, and complete lysis was confirmed microscopically. Homogenates were ultracentrifuged at 86000 × g for 45 min at 4°C. The supernatants were carefully removed and designated as the cytosolic fraction. Pellets were resuspended in containing 1% Triton X-100, incubated on ice for 30 min, and centrifuged at 14000 × g for 20 min at 4°C; the supernatants of this centrifugation step were designated as the membrane fraction.

Statistical analysis. Data were analyzed by using the unpaired Student’s t-test for analysis of the 2 groups, and Fisher’s protected least significant difference (Fisher’s PLSD) for analysis of multiple group comparisons. Results were expressed as the mean±S.D. P<0.05 was considered statistically significant.

Results

Co-expression of MMP-13 and PTHrP by bone metastatic breast cancer cells. While physiologic bone turnover involves cathepsin K and MMP-9 expression by osteoclasts and MMP13 expression by osteoblasts (21), it is not clear whether protease expression by bone-invading cancer cells themselves contributes to their metastatic behavior. To examine this question, we injected MDA321 breast cancer cells in nude mice intracardially and monitored the effects over time. Metastatic osteolytic lesions developed within 4 weeks from inoculation, with a preference for the distal femur and proximal tibia (Figure 1A). Close inspection of histological serial sections indicated that breast cancer cells had invaded into certain sites in the bone marrow (Figure 1B). MMP-13 and PTHrP were strongly co-expressed by the bone-invading cancer cells (Figure 1C-E). PTHrP

Figure 1. Distribution of MMP-13, PTHrP, and PTHIR in osteolytic bone metastasis in nude mice. A: Representative X-ray radiography of the hind limb of a mouse 5 weeks after it had been inoculated with MDA-MB-231 cells; arrowheads point to osteolytic lesions. B: Representative H&E-stained section of a distal femur bearing MDA-MB-231 cells. MMP-13 (C), PTHrP (D and G) and PTHIR (F) strongly characterize cancer cells in osteolytic bone metastasis. (E) The merged image showing a phase-contrast microscopic view of C and D. H: The merged image showing a phase-contrast microscopic view of F and G. Tm, Tumor cells; Bn, bone. Scale bar, 100 μm.
Figure 1F was also co-localized with PTH1R (Figure 1G) in the cancer cells (Figure 1H), supporting the idea that PTHrP may act in an autocrine manner on cancer cells in regions of bone metastasis. These results suggest that MMP-13 produced by the cancer cells may have directly contributed to their metastatic behavior and that its expression may have obeyed a PTHrP-dependent autocrine stimulatory loop.

Stimulation of MMP-13 expression by PTHrP. PTHrP-treated cultures contained much higher amounts of MMP-13 associated with the cell layer (Figure 2A) or released into the medium (Figure 2B) compared with the untreated control cultures, amounting to as much as a 3- to 4-fold increase at 100 nM PTHrP.

PKC and PKA involvement in MMP13 gene expression. To determine whether PKA or PKC had any roles in the PTHrP effects on MMP13 levels, we asked whether experimental activation of PKA or PKC would mimic the PTHrP effect. db-cAMP and TPA treatments resulted in a stimulation of MMP13 gene expression (Figure 3A and B). As seen in the above experiments, the stimulation was dose dependent and amounted to as much as 3-fold at the maximum treatment dose. To strengthen the data, we treated similar MDA-MB-231 cultures with 100 nM PTHrP for 8 h in the absence or presence of increasing concentration of H89 (a widely used inhibitor of PKA) or GF109203X (a widely used inhibitor of PKC). Treatment with the PKA inhibitor reversed the PTHrP-dependent stimulation of MMP13 gene expression, which actually dropped below the control value at 20 μM PKA inhibitor (Figure 3C). In a similar manner, PKC inhibitor treatment also significantly counteracted the PTHrP-dependent stimulation of MMP13 expression at the 20 μM concentration (Figure 3D).

ERK1/2 involvement in MMP13 gene expression. PD98059 reversed the PTHrP-induced stimulation of MMP13 gene expression, bringing sit down to the level seen in the untreated control cultures (Figure 4). In fact, the inhibitor also reduced MMP13 gene expression in cultures not exposed to PTHrP, indicating that even basal expression of this protease was at least partially dependent on the ERK1/2 pathway.
PTHrP effects on ERK and PKCα phosphorylation. Using immunoblot analysis with antibodies against active (phosphorylated, p) forms of PKCα and ERK1/2, we found that PTHrP treatment resulted in a time-dependent increase in the levels of p-PKCα and p-ERK1/2 (Figure 5A and C). Phosphorylation of PKCα increased as early as 1 min after the addition of 100 nM PTHrP and became maximal at 30 min, whereas that of ERK1/2 reached its maximum at 15 min after the addition of PTHrP (Figure 5C). Overall levels of PKCα and ERK1/2 remained constant during the 30-min period of incubation. We found also that PTHrP treatment induced a change in PKCα from a cytosolic to a membrane-bound status (Figure 5B), as well as causing p-ERK1/2 to be translocated from the cytoplasm to the nucleus (Figure 5D and E). Treatment with PD98059 reversed the PTHrP stimulation of p-ERK. Interestingly, treatment with GF109203X did the same; whereas TPA further increased the p-ERK level (Figure 6).

**Figure 3.** Modulation of PKA and PKC activities, and MMP13 gene expression. MDA-MB-231 cells were treated with increasing concentrations of dibutyryl-cAMP (db-cAMP, A) or 12-O-tetradecanoylphorbol-13-acetate (TPA, B) for 8 h. In addition, other cells were preincubated with increasing concentrations of H89 (C) or GF109203X (D) for 30 min and then treated with 100 nM PTHrP for 8 h. Total RNAs were analyzed by real-time RT-PCR. Asterisks indicate a significant difference at *p*<0.05.

**Figure 4.** Effects of inhibition of ERK1/2 on PTHrP-induced MMP13 gene expression. MDA-MB-231 cells were preincubated with increasing concentrations of PD98059 and then treated with 100 nM PTHrP for 8 h. Total RNAs were analyzed by real-time RT-PCR. GAPDH served as the internal control. Asterisks indicate significant difference at **p**<0.01.
Discussion

Breast cancer cells markedly induce the bone-resorbing activity and the expression of RANKL and MMP-13 in bone (22). Cancer cells employ a variety of mechanisms to elicit bone resorption. It is widely accepted that the most important of these is likely to involve the production of factors that activate osteoclasts, in particular PTHrP (6), and that osteoclast-mediated bone resorption is a major consequence of bone metastasis (23). Our data indicate that MDA-MB-231 breast cancer cells were present in very close association with the resorbing bone surface during metastasis and that PTHrP-expressing ones also highly expressed MMP-13. This observation suggests that the cancer cells themselves were acting as bone-resorbing cells and thus played a major and direct role in the invasive process. This interpretation is in line with the results of a recent study indicating that MMP-13 positively regulates mammary tumor-induced osteolysis (17), as well as with the up-regulation of MMP-13 seen in clinical sample of bone metastases from breast cancer patients (24). Our present data and the previous reports suggest an important role for the expression of MMP-13 at the tumor bone interface (22, 25, 26); hence, in this report, we focused on how MMP-13 expression was regulated by PTHrP in tumor-induced osteolysis.

PTHrP has been reported to regulate MAPK in several systems. PTHrP stimulates rat bone marrow cell proliferation through PKC-mediated activation of Ras/MAPK (27) and

Figure 5. PTHrP effects on PKCα and ERK1/2 activation. MDA-MB-231 cells were treated with 100 nM PTHrP for the indicated times, and cell layer extracts were then analyzed by immunoblotting with antibodies recognizing pPKCα and PKCα (A) or pERK1/2 and ERK1/2 (C). Bar graphs show the average of quantified data from 3 independent experiments. An asterisk denotes a significant difference (*p<0.05) from the control value. In parallel experiments, cells were serum-starved and treated with 100 nM PTHrP for 30 min. Some cultures were subfractionated into cytosolic and membrane-rich fractions and processed for immunoblot analysis of PKCα (B). Others were immunostained for intracellular distribution analysis of p-ERK in untreated (D) and PTHrP-treated cells (E).
induces osteosarcoma cell differentiation via PKA, PKC, and MAPK (28). It has also been reported that PTH stimulates MMP-13 expression through PKC and AP-1 (29). Activated ERK triggers the activation of AP-1 on the MMP13 promoter and contributes to cartilage destruction (30). The PKC signaling pathway has also been implicated in pathogenesis involving ECM remodeling and increased MMP-13 expression (31). Of note, a similar signal pathway has also been reported to be active in bone metastasis of MDA-MB-231 cells, which involves PTHrP-induced PKC-ERK-dependent expression of connective tissue growth factor (CTGF/CCN2) and VEGF (9, 20). In this study, we found that GF109203X blocked PTHrP-induced ERK1/2 activation and inhibited PTHrP-induced MMP13 gene expression in MDA-MB-231 cells. These results suggest that activation of PKC was required for ERK1/2 activation and that PTHrP-induced triggering of the PKC-ERK1/2 signaling pathway played a predominant role in MDA-MB-231 cell-induced bone metastasis.

In sum, our studies provide evidence that MMP-13 was strongly expressed by MDA-MB-231 cells as they metastasized to bone and suggest that MMP-13 expression at those lesion sites may reflect an autocrine loop involving PTHrP-PKA and -PKC-ERK1/2 signaling pathways. An increase in PTHrP expression coupled with its induction of bone-derived transforming growth factor-β (TGF-β) would favor tumor growth into bone (32). Our results additionally call attention to the possibility of therapeutically targeting these pathways to ameliorate the vicious cycle of osteolysis and further ingrowth during breast cancer metastasis.

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References


