Humanized Monoclonal Antibody Against Parathyroid Hormone-related Protein Suppresses Osteolytic Bone Metastasis of Human Breast Cancer Cells Derived from MDA-MB-231

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Abstract. Background: Parathyroid hormone-related protein (PTHrP) has been implicated in bone metastasis. However, the effects on bone metastasis of blocking the PTHrP function have not been tested in the clinic. Here, the effects of a humanized anti-PTHrP monoclonal antibody (mAb) on bone metastasis in a human xenograft model are shown. Materials and Methods: Subline MDA-5a, with high bone metastatic activity, was established from the human breast cancer cell line MDA-MB-231. Mice were injected with MDA-5a and an anti-PTHrP monoclonal antibody (mAb) raised against human PTHrP (1-34); bone metastasis was evaluated by X-ray photography. Results: MDA-5a produced elevated levels of PTHrP, Interleukin 8 (IL-8), IL-6 and matrix metalloproteinase 1 (MMP-1) and frequently metastasized to the bone. Administration of the humanized anti-PTHrP mAb significantly suppressed osteolytic bone metastasis of MDA-5a and caused osteogenesis at the sites of metastasis. Conclusion: The humanized anti-PTHrP mAb was effective against bone metastasis by inducing osteogenesis and, therefore, will provide a new treatment option for bone metastasis in breast cancer.

Breast cancer often metastasizes to the bone and causes severe bone pain and bone fracture (1, 2). Evidence suggests that metastasized cancer cells acquire characteristics distinct from those of the primary tumors (3). Several studies demonstrated that human breast cancer cells, with enhanced bone metastatic activity, expressed elevated levels of several cytokines, such as parathyroid hormone-related protein (PTHrP), Interleukin 8 (IL-8), matrix metalloproteinase 1 (MMP-1), chemokine receptor CXCR4, IL-11, and connective tissue growth factor (CTGF) (4-6), as compared to non-metastatic cells. Among these factors, PTHrP, known to activate osteoclasts, has been shown to play important roles in the bone metastasis of breast cancer cells (4, 7-9). In fact, a murine antibody raised against PTHrP was shown to suppress bone metastasis of breast cancer cells (8), and PTHrP was detected in two-thirds of over 100 human primary breast tumors (10). Nevertheless, the effects on breast cancer bone metastasis of blocking the PTHrP function have not been tested in clinical settings, simply because no humanized antibody has been available.

In a previous paper, we reported the generation of a humanized anti-PTHrP monoclonal antibody (mAb) raised against N-terminal 34 amino acids of the human PTHrP (11). Because binding sites to the receptor PTH1R resides in the N-terminal parts of PTHrP, this mAb was fully capable of blocking the binding of PTHrP to PTH1R (11). As a consequence, administration of the humanized anti-PTHrP mAb to rats bearing a human tumor xenograft that secreted PTHrP, thereby developing humoral hypercalcemia of malignancy (HHM), restored normal blood calcium levels (11). The efficacy of the humanized anti-PTHrP mAb against HHM in clinical settings is being evaluated.

In this study, the effects of the humanized anti-PTHrP mAb on bone metastasis of breast cancer were examined and it was shown that the humanized anti-PTHrP mAb suppressed osteolytic bone metastasis of breast cancer cells. Although bisphosphonate suppresses bone metastasis by inhibiting both bone resorption and formation (12, 13), the anti-PTHrP mAb induced osteogenesis at the sites of metastasis. Thus, the humanized anti-PTHrP mAb will
provide a new therapeutic option for the treatment of breast cancer patients with bone metastasis.

Materials and Methods

Animals. Female nude mice (BALB/c-nu/nu) were obtained from Clea Japan (Tokyo, Japan) and maintained under pathogen-free conditions throughout the experiment. The animals used in this experiment were treated in accordance with Chugai Pharmaceutical's ethical guidelines for animal care, handling and termination.

Cells. Human breast cancer MDA-MB-231 (MDA-231) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). To isolate cell fractions with bone metastasis activity, 10^6 cells/mouse of the MDA-231 cells were injected into the left ventricle of the hearts of female nude mice. Seven to 10 weeks after tumor inoculation, the MDA-231 cells at the sites of bone metastasis were recovered, expanded in the culture medium and again inoculated into the left ventricle of the hearts of female nude mice. By repeating the inoculation into mice and recovery from the sites of bone metastasis of MDA-231 cells 5 times, cells with high bone metastatic activity were selected and designated MDA-5a.

Administration of the anti-PTHrP mAb. A humanized anti-PTHrP antibody raised against human PTHrP (1-34) (11) was intravenously administered at the dose of 10 mg/kg twice a week, starting 7 days before tumor inoculation. Bone metastasis was evaluated by X-ray photography (Sofron, Tokyo, Japan) 49 days after tumor inoculation (day 49). The degree of osteolytic bone metastasis was determined using a grading system as follows: 0, no metastasis; 1, little and mild metastasis; 2, little and moderate metastasis; 3, abundant and moderate metastasis; and 4, abundant and severe metastasis. The right and left hind limbs were scored separately and a total score from 0 to 8 was given to each animal. Adrenal metastasis was confirmed microscopically at necropsy.

Histological examination. The hind limbs and adrenals of the mice were fixed in 10% formalin. After soaking in 20% EDTA, the specimens were embedded in paraffin. Sections were stained with hematoxylin and eosin or anti-PTHrP polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), as described (14).

ELISA and RIA assay. MDA-231 and MDA-5a cells were cultured in the presence or absence of humanized anti-PTHrP mAb for 24 hours and the amounts of hIL-6, hIL-8 and hMMP-1 in the conditioned media were determined by ELISA with BIOTRACK human IL-6, BIOTRACK human IL-8 and BIOTRACK human MMP-1 assay kits (Amersham Pharmacia Biotech). The detection limits of these assays were 10.24 pg/mL for hIL-6, 25.6 pg/mL for hIL-8 and 6.25 ng/mL for hMMP-1. The amounts of PTHrP were determined by RIA, as described elsewhere (15). The DNA contents of the cells were determined with a FluoroReporter Blue Fluorometric dsDNA Quantitation kit (Molecular Probes, Eugene, OR, USA), and the amounts of PTHrP, IL-6, IL-8 and MPP-1 were normalized against those of the DNA.

Statistical analysis. The results were analyzed with an SAS statistical package (version 6.12) and p-values less than 0.05 between the two groups were considered significant.

Results

Establishment of the cell line with high bone metastatic activity. When injected into the heart, MDA-231 cells frequently metastasized to the adrenals, but there were few metastasized tumors in the bone even 6 weeks after tumor inoculation.

To examine the effects of the humanized anti-PTHrP mAb on bone metastasis, the fractions of MDA-231 cells which efficiently metastasized to the bone were first isolated. After injection into the heart, MDA-231 cells that had metastasized to the bone were recovered, expanded in vitro and again injected into the heart. Repeating the inoculation and recovery of MDA-231 cells from the sites of bone metastasis increased the incidence of bone metastasis. By repeating the inoculation and recovery of the cells 5 times, a subline of MDA-231 was obtained and designated MDA-5a, in which cells possessing the ability to metastasize to bone were highly enriched (Figure 1A). The incidence of osteolytic bone metastasis from MDA-5a was significantly greater in number and size than from MDA-231 (Figure 1B), whereas the incidence of metastasis to the adrenals was not significantly different between the 2 cell lines (Figure 1C).

Gene expression profiling by DNA microarray revealed that PTHrP, IL-6, IL-8, IL-1β and MMP1, all of which have been considered to be involved in osteolytic bone metastasis (4-6, 8, 16), were up-regulated in MDA-5a more than in MDA-231. Therefore, the protein levels of PTHrP, IL-6, IL-8, IL-1β and MMP-1 in MDA-5a were examined by RIA or ELISA and the levels in the culture media were found to be several times higher in MDA-5a than in MDA-231 (Figure 2A), with the exception of IL-1β, which showed undetectable levels in both cell lines.

We also questioned whether increased levels of IL-6, IL-8 and MMP1 in MDA-5a depended on PTHrP. Cultivation of the MDA-5a cells in the presence of the humanized anti-PTHrP mAb did not affect the levels of
A

![Graph showing the percentage of mice with bone metastasis over the number of recovery cycles for MDA-231 and MDA-5a.]

B

![Images of bone lesions for MDA-231 and MDA-5a, labeled as 'No lesion', 'Bone resorption', 'Bone resorption', 'Bone fracture'.]

C

![Graph showing the percentage of mice with adrenal metastasis for MDA-231 and MDA-5a.]

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IL-6, IL-8 or MMP-1 in the culture media, indicating that the increased expressions of IL-6, IL-8 and MMP1 in MDA-5a occurred independently of the elevated level of PTHrP (Figure 2B).

Suppression of bone metastasis by the humanized anti-PTHrP mAb. In breast cancer patients, PTHrP expression was up-regulated in tumors metastasized to the bone more than in the primary tumor and metastasized to other organs (17). In addition, Guise et al. demonstrated that administration of the murine mAb against PTHrP suppressed bone metastasis (8). These facts raise the possibility that the bone metastatic activity of MDA-5a cells largely relies on an increased expression of PTHrP. Therefore, the levels of PTHrP in the tumor tissues of MDA-5a metastasized to the bone and those metastasized to the adrenals were examined using immunohistochemistry. At 4 weeks after tumor inoculation, the intensity of brown color derived from PTHrP was significantly stronger in the tumor tissues at the sites of bone metastasis than at the sites of adrenal metastasis (Figure 3). No brown color was observed from staining with a control human IgG (not shown). The result that PTHrP expression was up-regulated in tumor tissue in the bone microenvironment is consistent with observations in patients, indicating the clinical relevance of this animal model.

Next, the effect of humanized anti-PTHrP mAb on bone metastasis of MDA-5a was examined. The degree of bone metastasis was scored according to the number and size of the osteolytic bone lesions observed in X-ray photographs (Figure 4). Administration of the humanized anti-PTHrP mAb significantly suppressed bone metastases, but did not affect the incidence of metastasis to the adrenals (Figure 5A, 5B). Furthermore, histological examination revealed that administration of the anti-PTHrP mAb, but not of saline, induced osteogenesis caused by intramembranous ossification at the site of bone metastasis. Newly-formed bones showing an irregular shape, woven bone structures and osteoid
osteocytes were detected at the sites of metastasis in mice administered the anti-PTHrP mAb, whereas only osteoclasts and bone resorption were observed at the sites of metastasis in the saline-treated control mice (Figure 5C).

**Discussion**

In this study, we established MDA-5a, a subline of the breast cancer cell line MDA-231, by enriching the cell fractions for high bone metastatic activity. MDA-5a cells expressed an elevated level of PTHrP as compared to the original MDA-231 cells, and the humanized anti-PTHrP mAb suppressed the bone metastasis but not the adrenal metastasis of MDA-5a. Although the expressions of IL-6, IL-8 and MMP1 were also up-regulated in MDA-5a, the anti-PTHrP mAb did not affect the expressions of IL-6, IL-8, or MMP-1. At the site of osteolytic bone metastasis, enhanced bone resorption leads to the release of TGFβ from the resorbed bone, and the released TGFβ up-regulates the expression of PTHrP (18). Although the expression of TGFβ was not significantly increased in MDA-5a as compared to the parental MDA-231 in culture (not shown), it is likely that the PTHrP expression was further increased by TGFβ at the sites of bone metastasis. In addition, PTHrP might facilitate osteolytic

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**Figure 3.** Increased expression of PTHrP in tumor tissues metastasized to bone. Nude mice were injected with $10^5$ cells/mouse of MDA-5a and tumor tissues in the adrenals and bone were excised, fixed and examined histochemically. HE: Hematoxylin and eosin staining. IHC: Immunohistochemical staining with anti-PTHrP antibody.
Figure 4. Typical radiographs for the scoring of the osteolytic bone lesions. The degree of bone metastasis was graded according to the number and severity of osteolytic bone lesions. A score for each hind limb was given and the total from both hind limbs was determined. A typical example of each grade (0 to 4) is shown.

Figure 5. Suppression of bone metastasis by humanized anti-PTHrP antibody. Nude mice injected with 10^5 cells/mouse of MDA-5a were administered humanized anti-PTHrP mAb. Control mice received saline. A. The degree of bone metastasis determined from X-ray photography 7 weeks after tumor inoculation is shown. The values show the mean of 12 mice with standard errors. The asterisk indicates a significant difference (p<0.05) between the control group and the anti-PTHrP mAb-treated group. B. Incidence of metastasis to the adrenals, determined microscopically, is shown. C. Tumor tissues of MDA-5a at the sites of bone metastasis in mice receiving saline (left panel) or anti-PTHrP mAb (right panel) were excised and examined histochemically. Arrows (left panel) and asterisks (right panel) indicate areas of bone resorption with osteoclasts and newly-formed bones with osteoid osteocytes, respectively, within the sites of bone metastasis.
bone metastasis independently of IL-6, IL-8 and MMP1. In fact, blockade of the PTHrP function by the humanized anti-PTHrP mAb suppressed the bone metastasis of MDA-5a.

Administration of the humanized anti-PTHrP mAb not only suppressed the bone metastasis of MDA-5a, but also induced osteogenesis at the metastatic sites. Because bisphosphonate inhibits both bone resorption and bone formation (12, 13), osteogenesis by the anti-PTHrP mAb at the site of metastasis is presumably due to restoration of the activities of osteoblasts and other cells that are involved in bone formation. In fact, administration of the anti-PTHrP mAb increased, although only slightly, a bone formation marker in rats with bisphosphonate-refractory HHM (13). Elevated levels of PTHrP in tumor tissues metastasized to bone have also been observed in breast cancer patients (17) and, therefore, the humanized anti-PTHrP mAb will provide a new therapeutic option for the treatment of osteolytic bone metastasis in breast cancer.

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References


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