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Circulating interleukin-8 levels explain breast cancer osteolysis in mice and humans

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ABSTRACT

Skeletal metastases of breast cancer and subsequent osteolysis connote a dramatic change in the prognosis for the patient and significantly increase the morbidity associated with disease. The cytokine interleukin 8 (IL-8/CXCL8) is able to directly stimulate osteoclastogenesis and bone resorption in mouse models of breast cancer bone metastasis. In this study, we determined whether circulating levels of IL-8 were associated with increased bone resorption and breast cancer bone metastasis in patients and investigated IL-8 action *in vitro* and *in vivo* in mice. Using breast cancer patient plasma (36 patients), we identified significantly elevated IL-8 levels in bone metastasis patients compared with patients lacking bone metastasis ($p < 0.05$), as well as a correlation between plasma IL-8 and increased bone resorption ($p < 0.05$), as measured by NTx levels. In a total of 22 ER+ and 15 ER− primary invasive ductal carcinomas, all cases examined stained positive for IL-8 expression. *In vitro*, human MDA-MB-231 and MDA-MET breast cancer cell lines secrete two distinct IL-8 isoforms, both of which were found to stimulate osteoclastogenesis. However, the more osteolytic MDA-MET-derived full length IL-8(1–77) had significantly higher potency than the non-osteolytic MDA-MB-231-derived IL-8(6–77), *via* the CXCR1 receptor. MDA-MET breast cancer cells were injected into the tibia of nude mice and 7 days later treated daily with a neutralizing IL-8 monoclonal antibody. All tumor-injected mice receiving no antibody developed large osteolytic bone tumors, whereas 83% of the IL-8 antibody-treated mice had no evidence of tumor at the end of 28 days and had significantly increased survival. The pro-osteoclastogenic activity of IL-8 *in vivo* was confirmed when transgenic mice expressing human IL-8 were examined and found to have a profound osteopenic phenotype, with elevated bone resorption and inherently low bone mass. Collectively, these data suggest that IL-8 plays an important role in breast cancer osteolysis and that anti-IL-8 therapy may be useful in the treatment of the skeletal related events associated with breast cancer.

Introduction

Currently, there are approximately 2 million women in the United States living with breast cancer, and the disease is the second leading cause of cancer death in women [1]. Bone is a common site for cancer metastasis, and bone metastases are frequently associated with complications such as hypercalcemia due to osteolysis, nerve compression, intractable bone pain and pathological fractures [2]. Approximately 80% of women with metastatic breast cancer will have tumors arise in bone during the course of their disease [3].

The diagnosis of distant metastases mandates systemic rather than local primary intervention. Although largely incurable, patient management decisions regarding metastatic breast cancer suggest that initiation of early and aggressive treatment (both local and/or systemic) may be appropriate in some of these patients. The progression of breast cancer bone metastases requires the establishment of functional interactions between metastatic breast cancer cells and bone cells [4]. These interactions are presumably mediated by direct cell–cell contact and/or soluble stimulators that directly or indirectly induce osteoclast formation and activity [2,4–6].

In particular, breast cancer metastasis to bone presents unique characteristics which have been successfully targeted in the palliative

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setting, primarily by the use of bisphosphonates, which target the osteolytic activity of the osteoclast [2,5,6] and anti-RANKL therapy [2,5,7]. However, targeting the osteoclast alone does little to tumor burden in bone and suggests that other treatment modalities should be considered.

We and others have shown that the chemokine interleukin 8 (IL-8/CXCL8) is expressed by primary breast cancers and is able to directly stimulate osteoclastogenesis and bone resorption [8–11]. IL-8 is also a potent stimulator of the number and invasiveness of circulating tumor cells (CTCs) especially in tumors with enhanced bone tropism [12–15]. Collectively, these and other data suggest that IL-8 may play an important role in breast cancer progression [16]. One report suggested a correlation of serum IL-8 levels with breast cancer metastasis [17] and recently Singh and colleagues [18] suggested a role for IL-8 in the regulation of patient-derived breast cancer stem-like cell activity. However, the measurement of circulating IL-8 in breast cancer patients with and without bone metastasis and its correlation with levels of bone resorption has not been studied extensively.

Thus, we measured IL-8 in the circulation of breast cancer patients with and without bone metastasis and performed immunocytochemistry on a series of primary human breast cancers, as well as examined IL-8 function *in vivo* and *in vitro*. The data demonstrate that IL-8 is highly expressed by primary human breast cancers and is significantly correlated with elevated bone resorption in breast cancer patients. Tumor-derived systemic IL-8 contributes to increased tumor colonization, tumor growth and bone destruction, and these cellular events can be inhibited by anti-IL-8 therapy.

Materials and methods

Reagents

Tissue culture plastics were supplied by Falcon (Lincoln Park, NJ). All other analytical grade reagents were purchased from Sigma (St. Louis, MO) or Fisher (Springfield, NJ). All tissue culture media and reagents were supplied by Life Technologies, Inc. (Grand Island, NY). Recombinant human RANKL, recombinant murine macrophage colony stimulating factor (CSF-1), recombinant human IL-8, IL-8(1–77), and IL-8(6–77) and control IgG were purchased from R&D Systems (Minneapolis, MN). Anti-IL-8 antibodies used *in vivo* were obtained from R&D Systems.

Patient samples

Archival breast cancer patient plasma was obtained from 36 patients (18 with and 18 without bone metastasis) for the measurement of IL-8. Analysis of the archival plasma samples was approved by the University of Arkansas for Medical Sciences and Pennsylvania State University Institutional Review Boards. The clinical assessment of bone metastasis was based on patient bone scan, X-ray evidence of bone metastasis, and elevated blood N-telopeptide (NTx) levels, a clinical marker of bone resorption [19]. The serum NTx levels of all patients were used to help discern the presence or absence of bone metastasis. The women ranged in age from 49 to 92 years with a median age of 70 in the 'bone metastasis' group and 67 in the 'no bone metastasis' group. A power analysis was conducted to confirm that the size of the sample was sufficient to provide a statistical power of more than 80%.

In addition, a series of archival formalin-fixed paraffin embedded tumor tissue samples from 22 ER+ (expressing 2+ to 3+ positivity in >50% cells) and 15 ER− invasive ductal breast carcinomas, irrespective of grade and stage of disease, were also selected for evaluation. Unstained sections were immunostained for IL-8 expression (anti-IL-8 antibody, dilution 1:200, R&D Systems, Minneapolis, MN) with appropriate positive and negative controls. The intensity of staining for IL-8 was graded on a scale of 0 to 3+ with 0 representing no detectable staining and 3+ representing the strongest staining. Two independent observers examined each slide.

Cell lines and culture conditions

The MDA-MB-231 cells (MDA-231), MDA-MET cell lines and transfected variants (sense and anti-sense) were maintained in DMEM, supplemented with 10% fetal bovine serum at 37 °C in sterile culture dishes [9]. Highly bone metastatic MDA-MET cells were derived from a weakly osteolytic MDA-231 variant by *in vivo* selection [9]. MDA-MET cells secrete full length IL-8 and produce osteolytic lesions (100%) within 4 weeks of inoculation in the circulation or tibia of athymic nude mice [20] and grow effectively in the mammary fat pad [21] compared with MDA-231 cells that produce little full-length IL-8 [20].

MDA-231-IL8 and MDA-MET-AS cells were generated by stable transfection of expression vectors (pcDNA3 Invitrogen, Carlsbad, California) expressing full length hIL-8 or anti-sense hIL-8 cDNA as a *HindIII–BamHI* fragment by calcium phosphate precipitation. The pcDNA3/IL-8 sense or antisense (AS) DNA transfected cells were grown and single clones isolated by limiting dilution in the presence of the selective marker, G418 (Sigma Chemical Co., St. Louis, Missouri, USA). Clones were screened by measuring the amount of secreted hIL-8 (in sense expressing MDA-231 or the loss of IL-8 in MDA-MET antisense cells) in serum-free 48-h conditioned media. Clones with significantly increased and decreased IL-8 levels were selected for further study.

HEK-293 human embryonal kidney cells stably expressing CXCR1 or CXCR2 (a generous gift from Dr. Ji Ming Wang, NCI Frederick Cancer Research) were grown as monolayers in growth medium (Dulbecco's modified Eagle's medium with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml)). Cells were grown to approximately 75% confluency in an atmosphere of 95% air, 5% CO_2 at 37 °C as described [22].

IL-8 ligand binding assay

Ligand binding assays were performed as described [23]. Briefly, duplicate aliquots of stably transfected CXCR1 or CXCR2 HEK-293 cells (5×10^5–1×10^6 cells/200 μl) were re-suspended in binding medium (RPMI 1640; 10 mg/ml BSA; 25 mM HEPES; 0.05% Na azide) in the presence of 0.1 ng ^{125}IL-8 (DuPont-NEN, DE) and serial dilutions of unlabeled IL-8(1–77 or 6–77). Following 1 h incubation at room temperature the cells were centrifuged through 0.8 ml sucrose in PBS and the radioactivity remaining in the cell pellets was measured in a 1272 CliniGamma gamma counter (Pharmacia, MD).

Intratibial injection of tumor cells, antibody treatment and survival

MDA-MET, MDA-231-IL8 and MDA-MET-AS cells were grown to subconfluence. Fresh medium was added 24 h before harvesting for tumor inoculation. On the day of the injections, cells were harvested with 0.2% EDTA and 0.02% trypsin, washed twice in PBS and resuspended in PBS at a concentration of 10^6 cells/ml. Four-week-old athymic female nude mice were used for all tumor inoculations, purchased from Taconic Farms (Taconic, NY) and housed in an approved animal facility with protocols approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee.

Before injection, the animals were deeply anesthetized with a 1:1:4.6 solution of xylazine:ketamine:PBS (administered i.p. at 0.033-ml mixture/10-g body weight) as described [20]. The mice were placed in a prone position and after gently palpating the tibia, the upper end of the tibia was identified as the site for injection. Approximately 10,000 cells (in 10 μl) were injected into the right tibia of nude mice using a 50 μl syringe and 28 gauge needle. PBS only was injected in the left leg as vehicle control. After injection, the mice were placed on a heating pad to recover from anesthesia and then returned to their cages.

For the experiments using antibody treatments, animals were randomized into 3 groups: IL-8 antibody group (20 mice), control IgG