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Expression of Interleukin 8 and not Parathyroid Hormone-related Protein by Human Breast Cancer Cells Correlates with Bone Metastasis *in Vivo*[1]

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ABSTRACT

Metastasis is the process by which tumor cells spread from their site of origin to distant sites after gaining access to the circulatory system. An understanding of the factors contributing to the metastatic potential of breast cancer cells to bone will enhance the prospect of developing new therapies that impede metastasis. In this study, we have used an *in vivo* selection scheme involving left cardiac ventricle injection into nude mice to identify a highly metastatic human breast cancer cell line (MDA-MET) from a less metastatic (MDA-231) parental cell line. In this model, tumor-bearing mice exhibit features similar to those associated with human metastatic bone disease such as osteolytic bone destruction. After inoculation, MDA-MET cells form devastating lesions within 4 weeks, whereas the parental cells do not, even after 10 weeks. *In vitro*, the MDA-MET cells have a similar growth rate to the parental MDA-231 cells yet demonstrate distinct adhesive and invasive phenotypes. MDA-MET cells show increased early adhesion to type IV collagen and are significantly more invasive through Matrigel than MDA-231 cells. Analysis of the gene expression profile in the metastatic MDA-MET *versus* poorly metastatic MDA-231 cells identified relatively few genes whose expression was altered >2-fold. Of particular interest was the lack of parathyroid hormone-related protein (PTHrP) mRNA expression, which was supported at the protein level by immunoradiometric assay. These data support the idea that PTHrP is not predictive of the metastasis of human breast cancer to bone. Another important difference between the two cell lines was the elevated expression by MDA-MET cells of the cytokine IL-8. Reverse transcriptase-PCR and ELISA confirmed the increased expression of IL-8 in MDA-MET cells. In addition, IL-8 mRNA expression is also elevated in a variety of human cancer cell lines with different metastatic potential *in vivo*. These experiments suggest that the elevated expression of IL-8 (and not PTHrP) by MDA-MET cells is a phenotypic change that may be related to their enhanced ability to metastasize to the skeleton.

INTRODUCTION

Breast cancer is the most common malignancy in women worldwide with prevalence in the Western world of ~1.5 million (1). In the United States, the mortality rate is second only to lung cancer (2–4). Each year, nearly 180,000 women are diagnosed and nearly 44,000 women die of breast cancer. It is estimated that >80% of women dying with breast cancer have bony metastases (5, 6). Often osteolytic bone metastases cause devastating complications such as intractable bone pain, pathological fractures, hypercalcemia, and nerve compression syndromes, resulting in a significant decline in the quality of life of these patients. Despite the severity of these clinical problems and the need for effective treatment, therapies for metastatic breast cancer are currently often ineffective, stressing the urgency of understanding the underlying mechanism(s) of metastasis and developing new therapies.

To develop metastatic lesions, tumor cells must be able to accomplish each step in the multistep process while avoiding host immune surveillance (7). Two major factors determine the ability of cancer cells to disseminate to distant organs: the biological properties of the tumor cells and the environment at the metastatic site. It is generally accepted that cancer cells that metastasize to distant organs exhibit organ-specific characteristics that are often distinct from that of the primary tumor (7). Cancer cells with a high predilection to metastasize to bone therefore must have properties not present in tumors that rarely metastasize to bone (8).

Consistent with this idea, breast cancer cells that metastasize to bone have been shown to possess a number of altered properties such as increased PTHrP[3] production (9), elevated expression of PTH/PTHrP receptors (10, 11), and the expression of constitutively active estrogen receptor mutants (12). Additionally, a correlation between the expression of IL-8 and the metastasis of breast cancer cells, implanted in the mammary fat pad, to lung has been shown recently (13).

IL-8 is the prototypical member of a superfamily of small (M_r 8,000–10,000), inducible, secreted CXCs or α-chemokines that were originally identified as monocyte-derived factors capable of attracting and activating neutrophils (14, 15). A variety of cell types have been shown to synthesize and release IL-8 (and other cytokines) in response to injury, inflammation, and other pathological conditions (15). In addition, members of the α-chemokine family have also been observed as tumor cell products thought to contribute to the growth and progression of a large variety of tumor cells (16). Together, these results suggest that metastatic breast cancer cells have changed their phenotype (compared with nonmetastatic cells), such that they are now capable of colonizing other organs.

To test this hypothesis, we initiated an effort to identify populations of human breast cancer cells with different capacities to metastasize to bone. We used a heterogeneous population of MDA-MB-231 cells (referred to as MDA-231 human breast cancer cells; Ref. 17) with low metastatic potential and isolated via *in vivo* selection (7) a subpopulation of these cells (MDA-MET) with an enhanced metastatic potential. Using these two genetically related cell lines, we have begun to characterize their metastatic capacity, tumorigenicity, gene expression profile, and biological properties *in vitro* and *in vivo*.

MDA-MET cells form osteolytic bone lesions within 4 weeks compared with MDA-231 cells that do not form lesions even after 10 weeks. In this study, we have determined that the highly metastatic MDA-MET cells have enhanced early adhesion to type IV collagen and are more invasive through Matrigel. These properties are consistent with the increased metastatic phenotype observed *in vivo*. Microarray screening of the two cell lines has identified that the molecular fingerprint of gene expression in the metastatic *versus* nonmetastatic cells is remarkably similar. An important molecular difference between MDA-MET and MDA-231 cells may be the increased expression by MDA-MET cells of the cytokine IL-8, which

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[3] The abbreviations used are: PTHrP, parathyroid hormone-related protein; IL-8, interleukin 8; CXC, chemoattractant cytokine; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRMA, immunoradiometric assay; TGF-β, transforming growth factor β.

has been identified by gene expression profiling and independently validated by both reverse transcriptase-PCR and ELISA. The potential relevance of IL-8 expression in metastasis was demonstrated by its elevated expression in a variety of other human tumor cell lines with increased metastatic potential.

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).

Cell Lines and Culture Conditions

The MDA-MB-231 cells, MDA-MET, and MCF-7 (American Type Culture Collection, Manassas, VA) cell lines were maintained in DMEM, supplemented with 10% fetal bovine serum at 37°C in sterile culture dishes. The other human breast cancer cell lines MDA-435 and T47-D cell lines (American Type Culture Collection) were cultured in RPMI 1640, supplemented with 10% fetal bovine serum at 37°C and penicillin-streptomycin (Flow Laboratories, Rockville, MD). The human prostate tumor cell line PC-3 (American Type Culture Collection) was cultured in RPMI 1640, supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) at 37°C and penicillin-streptomycin (Flow Laboratories). All cell lines were certified to be *Mycoplasma* free. Cells were subcultured by trypsinization in 5 mg/ml trypsin (Sigma) and 0.5 mmol/liter EDTA in HBSS without calcium or magnesium in a laminar flow hood during their logarithmic phase of growth.

Animal Model

Subconfluent MDA-231 parental and MDA-MET cell lines were fed with fresh culture medium 24 h before harvesting for injection. Cells were harvested with 0.2% EDTA and 0.02% trypsin, incubated in culture medium (15 min), and suspended in PBS immediately before heart inoculation (0.1 million cells/0.1 ml PBS). All cell lines for injection were between the 3rd and 12th passage. Four-week-old athymic female nude mice were 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 cyanol:ketamine:PBS (administered i.m. at 0.033-ml mixture/10-g body weight). The mice were placed in a prone position and after drawback of fresh arterial blood was confirmed, the cell suspension slowly inoculated into the left cardiac ventricle using a 28 gauge needle. After injection, the mice were placed on a heating pad to recover from anesthesia and then returned to their cages. The development of osteolytic bone metastases was monitored by X-ray of anesthetized animals as described below.

X-ray and Histology

The development of bone metastasis (or tumor growth in bone) was monitored by X-ray of deeply anesthetized nude mice (4 weeks old at time of injection) using an AXR minishot 110 X-ray cabinet (Associated X-ray Corporation, East Haven, CT) at 3 mA, 33 kV for 20 s using Kodak X-Omat TL film (Kodak, Rochester, NY) and processed on a Kodak X-Omat RP automated film processor.

In parallel with the radiographic analysis, radiologically affected and unaffected limb long bones were excised, fixed in 10% neutral-buffered formalin for 2 days, and decalcified in 5% formic acid with agitation until deemed clear by the ammonium oxalate end point test (18). The decalcified specimens were then dehydrated through graded ethanol and cleared in methyl salicylate before paraffin infiltration. Subsequently, they were embedded in paraffin, sectioned (5 μm), and stained with H&E as described previously (18, 19).

Assays

Proliferation. *In vitro* proliferation of MDA-MET and MDA-231 cells was determined using the XTT assay (Sigma), which measures the mitochondrial activity of living cells as a readout for cell growth. Both cell lines were plated in 6-well multiwell dishes (50,000 cells/well), and proliferation was measured daily using the XTT assay, according to the manufacturers instructions.

Invasion. To perform the invasion assays, the upper chamber of the Transwell (coated with Matrigel) was incubated for 2 h at 37° and then seeded with 25,000 cells in DMEM containing 10% serum and 1% penicillin-streptomycin solution in a humidified atmosphere of 5% CO_2 in air. The upper chamber was then placed into the lower chamber that had been filled with 500-μl serum-containing DMEM. Transwell plates were then incubated for 4 and 24 h at 37°C. At the completion of the incubation period, culture media were carefully suctioned from the upper and lower chambers without disturbing the cells. The total number of invasive cells moving through the Transwell membrane was determined in triplicate by wiping the apical surface of the Transwell membrane with a cotton swab and staining the insert with Gill's hematoxylin 2 (Sigma), followed by fixation of the stain with 70% ethanol. The Transwell membrane was then cut out, mounted, and coverslipped on a microscope slide for cell counting. The total number of plated cells was evaluated at each time point by eliminating the cotton swab step and repeating the staining and membrane preparation. The percentage of invasive cells = (total cells on basilar surface)/(total number of cells on both apical and basilar surface). Cells were counted from multiple fields ($n = 6$) for each well (20× objective), with triplicate wells plated to ensure that adequate numbers of cells were counted (>1000 cells/cell line).

Adhesion. Cell attachment assays were performed according to the method of Thompson *et al.* (20) with minor modifications. The MDA-231 and MDA-MET cell lines were seeded into triplicate wells of 12-well multiwell dishes precoated with a variety of substrates (Becton Dickinson), including type IV collagen, and preincubated at 37°C for 2 h at 10,000 cells/well. Cells were then incubated at 37°C in 5% CO_2 for an additional 0.5, 2, 12, and 24 h. After incubation, medium was carefully removed from each well. Using a multichannel pipetter, each well was carefully washed three times with PBS. Between washes, each plate was gently rocked back and forth three times and the PBS carefully removed. After the third wash, the cells were stained with 0.05% crystal violet in 25% methanol for 15 min at room temperature to determine the number of remaining adherent cells (21, 22). The dye was removed and the plates gently rinsed with water and air-dried. The dye was then extracted with 1 ml of 0.1 M sodium citrate in 50% ethanol for 30 min with gentle shaking, and the absorbance measured at 540 nm. Previous experiments had demonstrated that absorbance is proportional to cell number (20, 21). The percentage of adherence for each time point = (mean number of cells remaining in well after washing)/(mean number of cells plated).

RNA Extraction

Total RNA was isolated from the *in vitro* tumor cell lines using the Qiagen RNeasy Midi kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Extracted RNA was quantitated by spectrophotometry and examined visually by agarose gel electrophoresis as described previously (23).

Microarray Hybridization, Analysis, and Assignment of Genes to Metastasis-associated Categories

As a first step in an effort to identify physiologically relevant gene expression in our clonally related breast cancer cell lines, we performed gene expression analysis using small, commercially available nylon arrays from both Clontech (Atlas blot, San Diego, CA) and Superarray (Bethesda, MD).

Total RNA was isolated from MDA-231 and MDA-MET cells as described above. Five to 10 μg of total RNA from each cell type were used for labeled cDNA synthesis using superscript II reverse transcriptase (Life Technologies, Inc., Rockville, MD) as recommended by the manufacturer. The relative expression level of a particular gene on each array was assessed using a phosphoimager (Molecular Dynamics, Sunnyvale, CA) after hybridization of the labeled cDNA to the arrays, according to the array manufacturer's specifications. Signal intensities for all genes were quantified and compared using ImageQuant software (Molecular Dynamics) after normalization to the signals for the housekeeping genes on both membranes as described previously (24). Genes were identified as regulated if the signal in MDA-MET was >2-fold different from MDA-231 cells.

We also performed more complex gene array analysis using Affymetrix GeneChips (Affymetrix, Santa Clara, CA). Double-stranded cDNA and bio-

tinylated cRNA were synthesized from total RNA (obtained from duplicate cultures of MDA-231 and MDA-MET cells) and hybridized to HuGeneFL GeneChip microarrays, which were washed and scanned according to the manufacturer's protocol. The arrays were scanned using a Hewlett Packard confocal laser scanner and visualized using Affymetrix 3.3 software (Affymetrix). Arrays were scaled to an average intensity of 1500 and analyzed independently.

GeneChip 3.3 output files were given (i) as an average difference that represents the difference between the intensities of the sequence-specific perfect match probe set, or (ii) as an absolute call of present or absent as determined by the GeneChip 3.3 algorithm. Statistical analyses of the data were performed using software packages as described previously (25).

In all, array comparison between MDA-MET and MDA-231 defined 126 of ~13,000 unique genes (<1%) that are differentially expressed. To focus our attention on groups of potentially important genes, we took a functional approach to determine the significance and relevance of these differentially expressed genes in highly metastatic (MDA-MET) *versus* low metastatic (MDA-231) breast cancer cells.

The differentially expressed genes were assigned to seven nominally exclusive metastasis-associated categories: proliferation; transcription; oncogenes; motility and cytoskeleton; immune surveillance; adhesion; and angiogenesis (26). Gene assignment to metastasis-associated categories was not mutually exclusive. The gene(s) represented in the most categories was selected for additional analysis. Verification of IL-8 gene expression (present in 5 of 7 categories) in MDA-231 and MDA-MET cells was performed by reverse transcriptase-PCR as described below.

Reverse Transcriptase-PCR

Total RNA was extracted from cell lines and reverse transcriptase-PCR analysis performed using specific human IL-8 primers. IL-8 primers were designed from the published human IL-8 sequence (Ref. 27; R&D Systems, Minneapolis, MN). GAPDH primers were synthesized as described previously (23). The 283-bp human IL-8 product was amplified using the following sequences: 5′-ATGACTTCCAAGCTGGCCGT-3′ and 5′-CCTCTTCAAAAACTTCTCCACACC-3′; and the 643-bp GAPDH product using the following primers 5′-ACGCATTTGGTCGTATTGGG-3′ and 5′-TGATTTTGGAGGGATCTCGC-3′. Each cycle set used a denaturing step (94°C, 60 s), annealing (55°C, 90 s), and extension (72°C, 90 s) for 20 cycles. Specific products were detected using a ^{32}P radiolabeled IL-8-specific internal probe. IL-8 mRNA was detected in both cell lines and appeared to be elevated ~3-fold in the MDA-MET cells compared with MDA-231 cells. The level of GAPDH mRNA detected by reverse transcriptase-PCR was similar in both cell lines.

IL-8 ELISA

The production and secretion of IL-8 by MDA-MET and MDA-231 cells were determined 1, 2, and 5 days hours after plating 1×10^5 cells in 300 μl of medium in 96-well plates. The supernatants of 4 wells from each time point and cell line were collected and analyzed for IL-8 expression using a commercially available ELISA kit (R&D Systems) according to the manufacturer's instructions. The commercially available IL-8 ELISA is specific for human IL-8. IL-8 concentrations in conditioned media were calculated from a standard curve generated by adding recombinant IL-8 to the specific unconditioned media and were considered undetectable if media concentrations were <0.3 pmol/liter before correction for cell number. Murine NIH 3T3 cells tested with the IL-8 kit did not secrete IL-8 cross-reacting products.

PTHrP IRMA

The production and secretion of PTHrP by MDA-MET and MDA-231 cells were determined in serum-free 24-h conditioned media using a commercially available PTHrP two-site IRMA (Nichols Institute, San Juan Capistrano, CA). The IRMA uses two polyclonal antibodies that are specific for the NH_2-terminal (1–40) and (60–72) portions of human PTHrP (28) and has a calculated sensitivity of 0.3 pmol/liter (29). PTHrP concentrations in conditioned media were calculated from a standard curve generated by adding recombinant PTHrP (1–86) to the specific unconditioned media and were considered undetectable if media concentrations were <0.3 pmol/liter before correction for cell number. The stimulation of PTHrP production by MDA-231 and MDA-MET cells cultured in the presence or absence of 10 ng/ml TGF-β (R&D Systems) was measured.

Statistical Analyses

Data are expressed as mean ± SE and analyzed by Student's *t* test using Microsoft Excel.

RESULTS

Derivation of Metastatic MDA-MET Cells

The MDA-231 cell line is an estrogen-independent human breast cancer line (Ref. 17; kindly provided by Dr. Tom Kelly University of Arkansas for Medical Sciences), which has been reported previously to metastasize to bone and other organs after left cardiac ventricle injection in nude mice (6, 30). The time frame for bone (and other organ) metastases with the MDA-231 cells in our hands is >10 weeks (Table 1), which is similar to the time course reported by others (30). The multiple sites of metastasis suggest that the MDA-231 parental cells consist of mixed subpopulations of breast cancer cells with different metastatic potentials.

To establish a cell line with an enhanced bone metastatic phenotype, MDA-231 cells in bone metastases were isolated by *in vivo* selection (31) and grown out from bone using the explant outgrowth procedure (Fig. 1; Ref. 32). During the *in vivo* selection process for bone metastasis, we also isolated a series of cell lines that had metastasized to other organs, including brain, as has been shown previously (Ref. 6; data not shown). The metastatic tumor cells from bone were isolated, grown in tissue culture, and then reinoculated into the left ventricle of female nude mice (30, 33). The cells that again metastasized to bone were isolated, expanded, and reinoculated into the left ventricle of the heart. This protocol was repeated until no micrometastases were detected histologically or by X-ray in tissues other than bone, within 4–5 weeks of injection (Table 1), as shown previously (6). The cells with enhanced metastasis to bone were termed MDA-MET cells (Table 1).

The MDA-MET cells were subsequently subcloned to a homogeneous population of metastatic cells, grown from a single cell (34), and are now routinely maintained in culture in our laboratory and are used for these studies only up to passage 12. In multiple subsequent experiments (~50 injected animals), no nonbone metastases have ever been observed after MDA-MET injection, even after 16 weeks (data not shown).

After reinjection of the clonal MDA-MET cells, a detailed histological analysis of the organs of injected mice at necropsy was performed. Multiple organs, including ovary, brain, liver, lung, kidney, spleen, and heart, were reviewed for evidence of metastasis by serial sections of fixed, paraffin-embedded tissue. Metastatic tumors were never seen in tissues other than bone. Similar observations have been made for other bone-seeking metastatic cell lines (6).

Table 1 *Establishment of the bone selectivity of MDA-MET cells*

Parental MDA-231 cells were passaged *in vivo* through nude mice. With sequential passage *in vivo*, fewer tumors were evident at nonbone sites. By passage 5, the clonal MDA-MET cell line yielded only bone tumors in 100% of mice (5 of 5), with no other organ involvement (0 of 5), even after 10 wk. In subsequent experiments with MDA-MET cells (currently ~50 animals), no metastatic tumors are seen in tissues other than bone.

Passage no.	Mice with bone metastasis (time to metastases)	Other organs (time to metastases)
1 (MDA-231)	4 of 5 (6–8 wk)	3/5 (8–10 wk)
2 (Harvested from bone)	4 of 5 (4–6 wk)	2/5 (>8 wk)
3 (Harvested from bone)	5 of 5 (4–6 wk)	(2/5) (>10 wk)
4 (Harvested from bone)	5 of 5 (4–6 wk)	(0/5) (>10 wk)
5 (Clonal line) (MDA-MET)	5 of 5 (4–5 wk)	(0/5) (>10 wk)

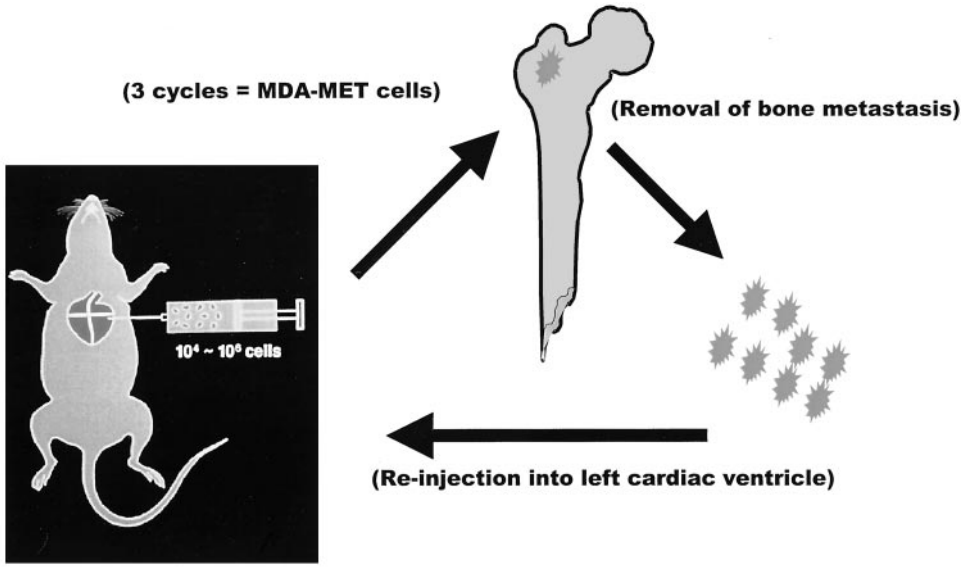

Fig. 1. Schematic representation of the *in vivo* selection process used to derive the highly metastatic MDA-MET cells. To establish a cell line with an enhanced bone metastatic phenotype, MDA-231 cells in bone metastases were isolated by *in vivo* selection and grown out from bone using the explant outgrowth procedure. The isolated bone metastasis tumor cells were grown in tissue culture and reinoculated into the left ventricle of female nude mice. The cells that again metastasized to bone were isolated, expanded, and reinoculated into the left ventricle of the heart. This protocol was repeated three times until no micrometastases were detected histologically or by X-ray in tissues other than bone, within 4 weeks of injection.

The osteolytic metastasis phenotype of the MDA-MET cells was confirmed by histology and X-ray after injection into the heart (Fig. 2). Four-week-old female nude mice were inoculated with MDA-231 or MDA-MET cells into the left cardiac ventricle. Four weeks after inoculation of MDA-MET cells, clear osteolytic bone destruction was observed (Fig. 2*A*). However, at the same time point after MDA-231 inoculation, no bone destruction was seen (Fig. 2*B*).

Similarly, mice were sacrificed 4 weeks after inoculation and the tibia excised and processed for conventional histological examination. Tumor infiltration into the proximal tibia was evident (Fig. 3*A*), with little bone marrow cavity remaining. Numerous osteoclasts were seen at the endosteal bone surface (Fig. 3*B*, *arrows*). At this time point, no tumor is visible in bone (or other organs) of MDA-231-injected animals (data not shown).

Expression of PTHrP Does Not Correlate with the Metastatic Phenotype of MDA-MET Cells

It is well recognized that breast tumors with the capacity to invade and grow as metastases in bone have a number of phenotypic properties that contribute to this feature. The expression of bone resorbing factors such as PTHrP has been shown to be an important feature of metastatic breast cancer cells (6, 30, 35). PTHrP is a potent osteoclast activating factor, originally identified as the cause of the humoral hypercalcemia of malignancy (28), that is expressed by two-thirds of all primary breast tumors (36). Therefore, given the wealth of studies correlating elevated PTHrP expression with the enhanced osteolytic phenotype of breast cancer cells *in vivo* (6, 8, 9, 10, 30, 35–37), we examined PTHrP expression in both the MDA-231 and MDA-MET cell lines.

PTHrP expression in MDA-231 and MDA-MET cells was measured using a two-site IRMA (Nichols Institute). The level of PTHrP protein secreted by the cells into the conditioned media/100,000 cells was 1.5 ± 0.3 pmol/liter (MDA-231) and 2.0 ± 0.4 pmol/liter (MDA-MET; Fig. 4). The secreted PTHrP levels measured by IRMA appear to correlate well with the mRNA levels in both cell lines (data not shown). Interestingly, treatment of MDA-MET cells with 10 ng/ml TGF-β did not increase PTHrP levels in 24-h conditioned media (Fig. 4). However, the same TGF-β treatment significantly

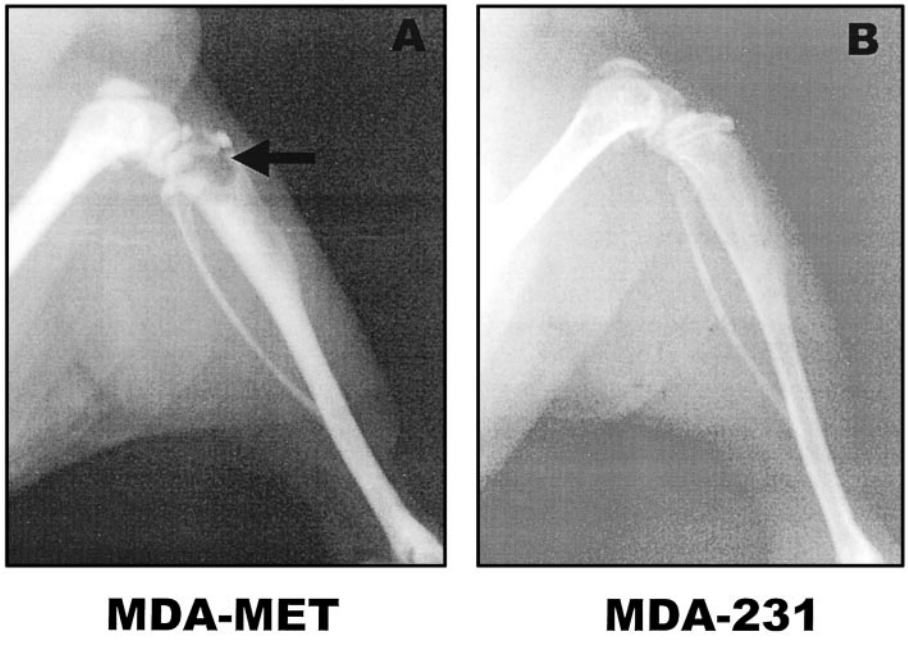

Fig. 2. The development of bone metastases *in vivo* was monitored by X-ray. Four weeks after MDA-MET inoculation (*A*), clear osteolytic bone destruction was observed (*arrow*). No bone destruction was seen with MDA-231 cells at the same time point (*B*).

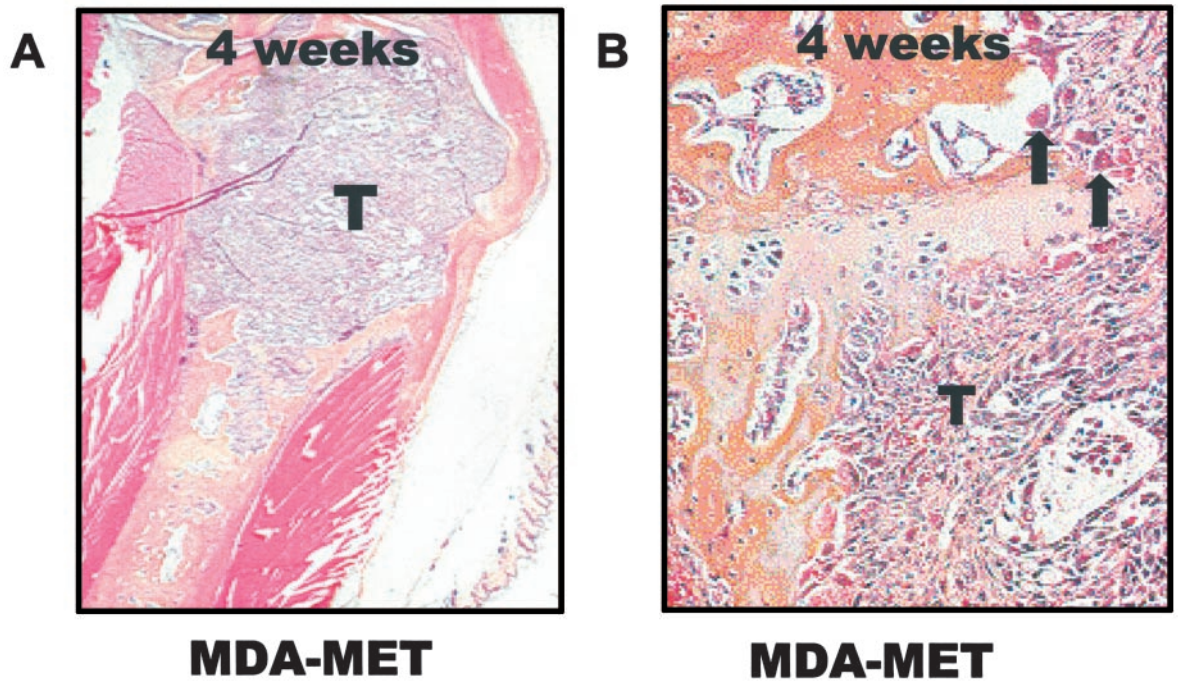

Fig. 3. Histological view of MDA-MET tumor in bone. Four-week-old female nude mice were inoculated with MDA-MET cells into the left cardiac ventricle. Four weeks later, mice were sacrificed and tibia excised and processed for conventional histological examination. *A*, tumor infiltration into the proximal tibia is evident (*T*) with little bone marrow cavity remaining (H&E staining, ×20). *B*, numerous osteoclasts are seen at the endosteal bone surface (*arrows*). (H&E staining, ×200).

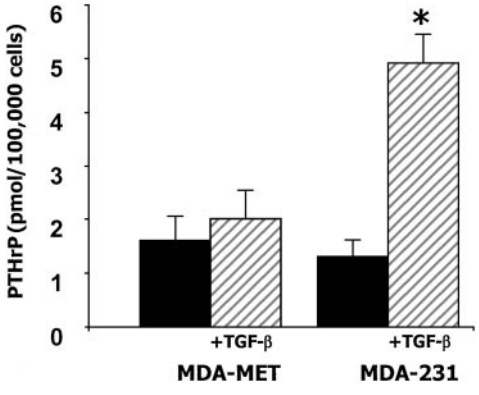

Fig. 4. PTHrP production by MDA-231 and MDA-MET cells in the presence or absence of TGF-β. MDA-231 and MDA-MET cells were grown to confluence in 12-well plates, washed with serum-free medium, and incubated overnight in serum-free medium in the absence (■) or presence (▨) of 10 ng/ml TGF-β1. The culture media were harvested, centrifuged to remove cell debris, and stored at −20°C until assay. PTHrP production was measured by IRMA. *, significantly different from untreated MDA-231 at $P < 0.05$. Results are the means ± SE ($n = 3$). Similar results were obtained from three separate experiments.

increased PTHrP expression in MDA-231 cells (Fig. 4) as shown previously (6).

Importantly, the lack of regulation of PTHrP expression by TGF-β in MDA-MET cells was confirmed by detailed microarray analysis (Table 2). Gene expression profiling identified a down-regulation of the ligand binding type II TGF-β receptor expression (38) in MDA-MET, an increase in smad 2 mRNA, as well as a lack of PTHrP mRNA (Table 2). These gene expression data support the lack of TGF-β stimulation of PTHrP observed *in vitro* (Fig. 4).

The fact that the dramatic *in vivo* bone metastatic phenotype of MDA-MET cells was not correlated with a marked overexpression of PTHrP (or regulation by TGF-β) prompted the interrogation of the *in vitro* phenotype of the two genetically related yet phenotypically distinct cell lines.

In Vitro Characterization of MDA-231 and MDA-MET Cells

Proliferation. To determine whether the distinct *in vivo* phenotype of MDA-MET cells was related to an increased intrinsic proliferative capacity of MDA-MET cells compared with MDA-231 cells, we evaluated the growth rate of the two cell lines *in vitro*. As shown in Fig. 5*A*, no difference in the growth rate between the two cell lines was observed.

Invasion through Matrigel. The invasive phenotype of both the MDA-231 and MDA-MET cells was assessed by comparing their relative abilities to invade through tumor extracellular matrix (Matrigel; Collaborative Biomedical Products, Bedford, MA) after 24 h,

Table 2 *Microarray analysis of TGF-β signaling pathway*

Genes known to be associated with the TGF-β signaling pathway, along with the genes for PTHrP and its specific receptor, were clustered and analyzed for absolute call (A, absent; P, present) or difference call between MDA-231 and MDA-MET (NC, no change; D, decreased; I, increased). The bolded genes (***TGF-β IIR α; human mad protein homolog smad 2 gene; PTH-related protein***) explain the low level expression of PTHrP and the lack of regulation by TGF-β. The microarray data confirm the *in vitro* analysis of PTHrP secretion presented in Fig. 4.

Gene name	Absolute call	Difference call
TGF-β mRNA	A	NC
mRNA for TGF-β	A	NC
TGF-β mRNA complete cds	P	NC
TGF-β 2	P	NC
TGF-β 3	A	NC
TGF-β-activated kinase 1a	P	NC
TGF-β-interacting protein	P	NC
mRNA for TGF-β superfamily protein	P	D
TGF-β R Type 1	P	NC
TGF-β IIR-α	P	D
TGF-β IIIR	P	NC
Human mad protein homolog smad 2 gene	P	I
Smad 3	P	NC
Smad 4		
Smad 6	P	NC
Smad 7	P	NC
PTHrP	A	NC
PTH/PTHrP receptor	A	NC

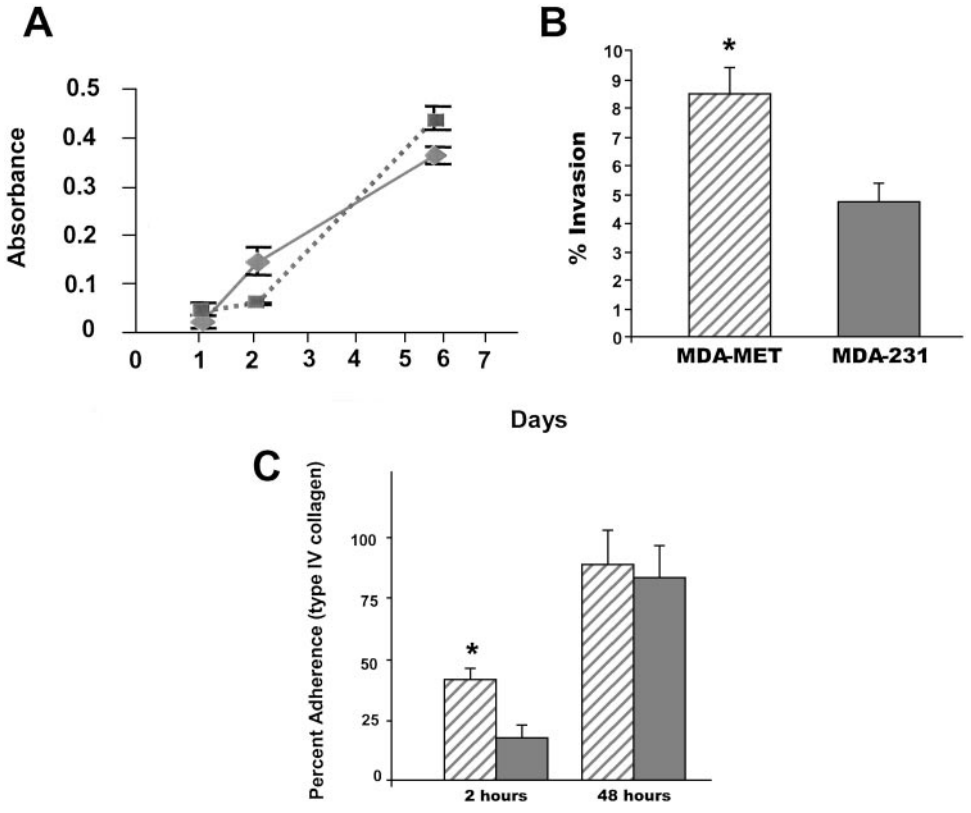

Fig. 5. *In vitro* phenotype of MDA-231 and MDA-MET cells. *A*, MDA-231 and MDA-MET cells were seeded (50,000 cells/well) in 6-well multiwell dishes, and proliferation was measured daily using the XTT assay as described in "Materials and Methods." ———, MDA-231 cells; ····, MDA-MET cells. *B*, MDA-231 and MDA-MET cells were incubated in the invasion assay as described in "Materials and Methods." Cells (■, MDA-231; ▨, MDA-MET) were counted in multiple fields ($n = 6$) for each well (20× objective), with wells plated in at least triplicate serve to ensure that adequate numbers of cells were counted (>1000 cells/cell line). ∗, significantly different from MDA-231 invasion at $P < 0.05$. *C*, differences in the heterotypic adhesion (cell-to-substrate adherence) of MDA-231 and MDA-MET were examined using collagen IV as a substrate. Adhesion was assessed at early (2 h) and late time points (48 h) as described in "Materials and Methods." After 2 h, the adhesion to type IV collagen is greater in MDA-MET cells (▨) than MDA-231 cells (■). No significant difference in adhesion to type IV collagen was observed between MDA-231 and MDA-MET cells at the 48-h time point. ∗, significantly different from MDA-231 adhesion at 2 h at $P < 0.05$. Results are the means ± SE ($n = 6$). Similar results were obtained from five separate experiments.

using the Transwell culture system (Costar, Cambridge, MA). As expected, both cell lines were invasive through Matrigel; however, MDA-MET invasion and migration through Matrigel were significantly greater than MDA-231 (Fig. 5*B*).

Extracellular Matrix Adhesion. Differences in the heterotypic adhesion (cell-to-substrate adherence) of MDA-231 and MDA-MET were examined using collagen I, collagen IV, fibronectin, and laminin as substrates. Adhesion was assessed at early (0.5 and 2 h) and late time points (24 and 48 h). The ability to initiate early adherence has been described as a distinct process from the maintenance of adhesion by cancer cells (26).

Differences in the adhesion of either cell type to any substrate were not evident at the earliest time point (0.5 h; data not shown). However, after 2 h, the adhesion to type IV collagen appeared greater in MDA-MET cells compared with MDA-231 cells after 2 h (early adhesion; Fig. 5*C*). However, no significant difference in adhesion to any substrate was observed between MDA-231 and MDA-MET cells at any of the later time points (maintenance of adhesion).

Microarray Analysis and Validation of Gene Expression

Microarray experiments examined differences in gene expression between the two related cell lines with distinct *in vivo* phenotypes, MDA-231 (low metastatic potential), and MDA-MET (aggressive metastatic phenotype). One hundred twenty-six of ~13,000 unique genes examined were identified as differentially expressed.

To focus our attention on groups of potentially important genes, we took a functional approach to determine the significance and relevance of these differentially expressed genes in highly metastatic (MDA-MET) *versus* low metastatic (MDA-231) breast cancer cells. By a review of the literature, we assigned each of the 126 differentially expressed genes to seven nominally exclusive metastasis-associated categories: proliferation; transcription; oncogenes; motility and cytoskeleton; immune surveillance; adhesion; and angiogenesis (26).

On the basis of this metastasis-related characterization, IL-8 was assigned to the most categories (5 of 7). On the basis of this categorization, IL-8 was considered most likely to be associated with the aggressive behavior of MDA-MET *in vivo* compared with MDA-231 cells.

The differential expression of IL-8 mRNA between MDA-MET and MDA-231 cells originally observed by microarray analysis was independently confirmed at both the RNA and protein levels (Fig. 6). IL-8 mRNA expression in both MDA-231 and MDA-MET cells was examined by reverse transcriptase-PCR analysis; protein secretion was measured in serum-free conditioned media using an ELISA assay (R&D Systems).

IL-8 mRNA was elevated in MDA-MET cells compared with MDA-231 cells (Fig. 6*A*), with little or no change in the level of GAPDH. Significantly, the level of IL-8 protein secreted by the cells in conditioned media/μg protein was 3–4-fold higher in MDA-MET cells compared with MDA-231 cells (Fig. 6*B*). IL-8 expression appeared to increase with time, reaching maximal levels between days 2 and 5. The secreted IL-8 levels measured by ELISA appear to correlate well with the mRNA levels determined by reverse transcriptase-PCR in both cell lines.

IL-8 Expression Correlates with Enhanced Metastatic Phenotype

To determine whether a correlation existed between IL-8 mRNA expression by the tumor cell line and their reported metastatic potential *in vivo* (Fig. 7), we tested a number of human cancer cell lines with different metastatic potential. Human breast cancer cell lines with little or no metastatic capacity (MCF-7, T47-D, MDA-231, and MDA-435), highly metastatic MDA-MET cells, and a well-characterized prostate tumor cell line (PC-3) with high metastatic potential (39) were screened for IL-8 expression by reverse transcriptase-PCR.

Reverse transcriptase-PCR analysis demonstrated that the human tumors cell lines with an increased capacity to metastasize (MDA-

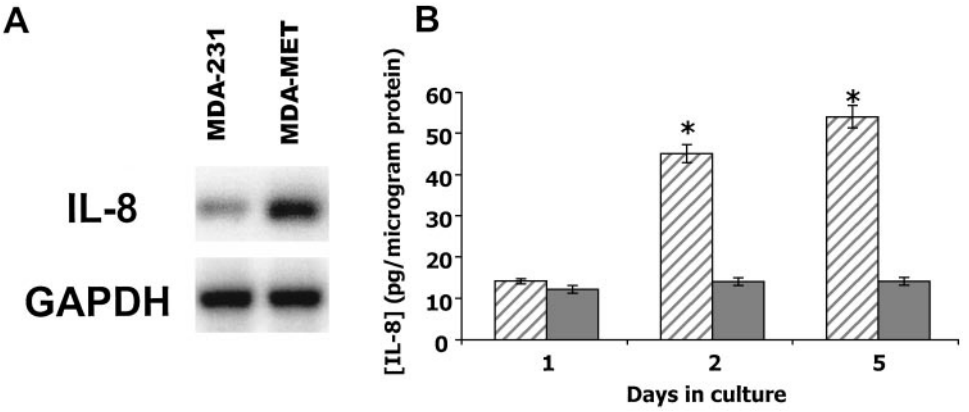

Fig. 6. IL-8 production by MDA-MET and MDA-231 cells. *A*, reverse transcriptase-PCR analysis of IL-8 mRNA expression in MDA-231 and MDA-MET cells. Elevated levels of IL-8 mRNA expression are seen in MDA-MET cells. No significant differences in GAPDH mRNA levels were detected. Similar results were obtained from three separate experiments. *B*, time course of IL-8 production by MDA-231 and MDA-MET cells. MDA-231 and MDA-MET cells were plated in 96-well plates and supernatants from each time point (days 1, 2, and 5) were collected and analyzed for IL-8 expression by ELISA as described in "Materials and Methods." IL-8 production increased over time in MDA-MET cells (▨) but not in MDA-231 cells (■). ∗, significantly different from untreated (day 1) MDA-MET at $P < 0.05$. Results are the means ± SE ($n = 4$). Similar results were obtained from three separate experiments.

MET and PC-3) also have a higher level of IL-8 mRNA expression (Fig. 7). The assignment of the metastatic potential of the MDA-435, T47-D, MCF-7, and PC-3 cell lines was made based on their reported ability to metastasize in immunosuppressed nude mice (13, 39). The level of GAPDH mRNA detected by reverse transcriptase-PCR was similar in all cell lines (Fig. 7), and in the absence of reverse transcriptase, no PCR products were detected (data not shown).

DISCUSSION

The observation that the majority of cancers exhibit target organ preference when they disseminate was first described in a study of the autopsy records of 735 women who died of breast cancer (40). On the basis of these seminal observations, Paget (40) proposed the "Seed and Soil" hypothesis, which has subsequently been widely accepted as a basic principle in the field of cancer metastasis (7). However, despite the enhanced ability of breast cancer to metastasize to the skeleton (5, 41–43), the precise mechanisms underlying this predilection remain poorly understood.

In an effort to investigate this pathway, we derived a cell line with an aggressive bone metastatic phenotype by repeated passage of the less metastatic MDA-231 cells *in vivo*. This *in vivo* selection process (6, 7, 44) generated a cell line with an enhanced bone metastatic phenotype, MDA-MET. This cell line exhibits differences in *in vitro* properties and displays a distinct *in vivo* phenotype compared with the parental cell line. With repeated inoculation of the clonal MDA-MET cells into the left ventricle of nude mice (currently ~50 animals), we have never observed metastatic tumor in any organ other than bone. We hypothesize that these altered phenotypic properties, which include enhanced invasion through basement membrane, early adhesion to type IV collagen, a lack of TGF-β-stimulated PTHrP secretion, and elevated expression of IL-8 are responsible for the osteolytic metastases observed after intracardiac injection of MDA-MET cells.

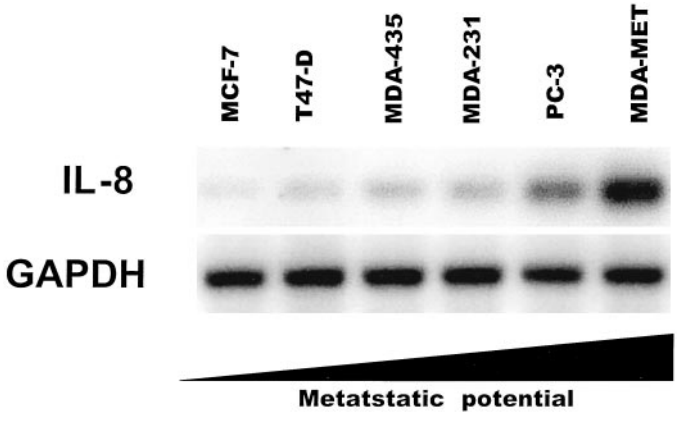

Fig. 7. IL-8 mRNA expression correlates with increased metastatic potential. Reverse transcriptase-PCR analysis of IL-8 mRNA expression in a panel of human cancer cell lines. The *in vivo* metastatic potential of the cell lines increases as shown. All cell lines are human breast cancer cells, except PC-3, which is a human prostate carcinoma cell line. Elevated levels of IL-8 mRNA expression correlates with increased metastatic potential. MDA-MET cells, with the highest metastatic potential, have the highest level of IL-8 mRNA expression. No significant difference in GAPDH mRNA level was detected. Similar results were obtained from two separate experiments.

A major barrier to the understanding of the metastatic process is the heterogeneity of cancer cells (31). This tremendous heterogeneity, which may be exhibited in a wide range of genetic, biochemical, immunological, and biological characteristics, is because of different etiologies, origins, and selection pressure placed on different cancers. Our search for factors involved in mediating the metastatic cascade of breast cancer to bone was greatly facilitated by the derivation of MDA-MET cells. The similar genotype, but distinct *in vivo* phenotype, between the related MDA-MET and MDA-231 cells allowed the use of cDNA microarray analysis to define potentially important genes involved in the bone metastasis that is characteristic of this model.

Using microarray analysis, genes that are differentially expressed in highly metastatic (MDA-MET) *versus* low metastatic (MDA-231) breast cancer cells were identified. These approaches have been successful recently in listing patterns of gene expression in a variety of cancers (26, 45–47). However, it remains difficult to determine how to best use this information.

In the cDNA microarray comparisons presented herein, 126 genes that were differentially expressed between the high (MDA-MET) and the low (MDA-231) metastatic breast cancer cell lines were identified. Using a simple reductionist approach to focus attention on the phenotypic property of bone metastasis, which is significantly different in the two related cell types, each of the 126 differentially expressed genes was assigned to nonmutually exclusive metastasis-related process categories (26). A total of seven process categories (proliferation, transcription, oncogenes, motility and cytoskeleton, immune surveillance, adhesion, and angiogenesis) were developed. This rationale assumed that if a gene was represented in multiple metastasis process categories, then that gene was more likely to play a significant role in the aggressive phenotype of the MDA-MET cells. The rate of assignment of the candidate genes is presumably related to the design of the comparison (using cells with a similar genotype but distinct metastatic potential) and the bias of the method used to assign the genes (PubMed search). Despite these caveats, we believe that this methodology represents a rational approach for the evaluation of microarray data that will be useful for the identification of additional genes causally associated with the metastatic process. We subsequently applied this approach to a larger more complex Affymetrix microarray

analysis of the two cell lines. Interestingly, microarray analysis did not detect elevated expression of PTHrP mRNA in MDA-MET cells, the factor largely considered to be the major determinant of bone metastatic potential (6, 10, 30).

The basal level of PTHrP expression in our MDA-231 cells is similar to that reported in MDA-MB-231 cells by Yoneda *et al.* (6). In that study, TGF-β treatment (5 ng/ml) increased PTHrP expression in both the MDA-MB-231 parental cells (to ~4 pmol/liter) and the bone-seeking clone (MDA-231BO; to ~10 pmol/liter). In other experiments, using MCF breast cancer cells inoculated into the left ventricle of nude mice, PTHrP overexpression of the order of ~1200 pmol/liter (35) was required to develop a metastatic phenotype similar to that seen in MDA-MET cells expressing only 2.0 pmol/liter of PTHrP. Taken together, these data suggest that the PTHrP secreted by MDA-MET cells is not sufficient to explain the marked metastatic difference between the two cell lines *in vivo*. These data also support our contention that factors other than PTHrP overexpression (and its regulation by TGF-β) may explain the phenotypic difference between MDA-231 and MDA-MET cells.

This idea is supported by a recent prospective clinical study of >400 women with operable primary breast cancer followed for a median period of 67 months (48). In that study, it was suggested that women with PTHrP-positive primary breast cancers have a more favorable outcome with fewer bone metastases (48). These important observations do not exclude a central role for PTHrP in bone metastasis; rather they emphasize the role of the bone microenvironment in eliciting local elevations in PTHrP production (1, 48). Collectively, these data lead us to speculate that MDA-MET cells are an excellent model of bone metastasis because, as in human disease, PTHrP expression in MDA-MET cells does appear to correlate with bone metastatic potential.

The role of TGF-β (and regulation by PTHrP) in the process of bone resorption adds even greater complexity. TGF-β is sequestered throughout the bone matrix and certainly released during osteoclast-mediated bone resorption (49). Several studies using intracardiac injection of breast cancer cells have suggested how TGF-β in the bone microenvironment contributes to tumor invasion by stimulating PTHrP production by the invading breast cancer cells (6, 10, 30). However, our microarray analysis, as well as *in vitro* evaluation of TGF-β-stimulated PTHrP production by MDA-MET breast cancer cells, suggests that a TGF-β-mediated signal cascade is not responsible for the enhanced bone metastatic potential of MDA-MET cells. Presumably, the release of TGF-β during tumor osteolysis *in vivo* does produce increases in local TGF-β production that stimulate PTHrP expression in nonbreast cancer cells, thereby amplifying bone resorption and supporting MDA-MET tumor growth in bone.

On the basis of microarray screening and our metastasis-related characterization of gene expression, the gene considered to be the most likely candidate to be associated with the aggressive bone metastatic behavior of MDA-MET compared with MDA-231 cells was IL-8.

IL-8 is proangiogenic and a potent chemoattractant, which has been associated with the enhanced cell motility, invasion, and metastatic potential of a number of human tumors (50). The elevated expression of IL-8 was confirmed at the RNA level using multiple RNA preparations and at the secreted protein level by ELISA.

IL-8 mRNA expression was also evaluated in six human tumor cell lines (five breast and one prostate) to determine whether a correlation existed between IL-8 mRNA expression by the tumor cell line and their reported metastatic potential *in vivo*. We tested human breast cancer cell lines with little or no metastatic capacity (MCF-7, T47-D, MDA-231, and MDA-435), the highly metastatic MDA-MET cells, and a well-characterized prostate tumor cell line with high metastatic potential, PC-3 (39). The assignment of the metastatic potential of the MDA-435, T47-D, MCF-7, and PC-3 cell lines was made based on their reported ability to metastasize in immunosuppressed nude mice (13, 39). The level of expression of IL-8 correlated exactly with the reported metastatic phenotype of the cell lines, identifying a potential important link between IL-8 expression and the metastatic potential of breast cancer cells.

A variety of cell types have been shown to synthesize and release IL-8 (and other cytokines) in response to injury, inflammation, and other pathological conditions (15). IL-8 production can be induced by proinflammatory cytokines (IL-1 and tumor necrosis factor α; Refs. 15, 51, 52). In addition to serving as a potent chemoattractant and activator of neutrophils, IL-8 also exerts a diverse array of physiological effects on target cells, including the stimulation of hematopoiesis, angiogenesis, and the rapid induction of changes in cell surface adhesion molecules such as integrins (15, 51, 53).

In bone, IL-8 is synthesized by osteoclasts (52) and osteoblasts (54) and has been shown to directly inhibit osteoblast alkaline phosphatase activity (54). IL-8 has also been shown to decrease bone resorption by increasing osteoclast motility to new resorption sites, rather than directly inhibiting bone resorption (55). However, such a process in the case of MDA-MET tumor-bearing animals would increase the demineralized surface exposed for colonization by tumor cells.

The biological actions of IL-8 are typically mediated by increases in intracellular calcium, which occur after binding to specific cell surface receptors, namely CXCR1 and/or CXCR2 (15). Interestingly, we have been unable to identify specific IL-8 receptors in MDA-231 and MDA-MET cells by microarray analysis, reverse transcriptase-PCR, or fluorescence-activated cell sorter (data not shown), suggesting that the effects of IL-8 in bone metastasis are not autocrine/paracrine in nature. Our data support the hypothesis that secreted IL-8 acts on cells distant from the secreting tumor, such as bone lining cells, osteoblasts, and/or other marrow components, to increase osteoclastogenesis, perhaps by an up-regulation of receptor activator of nuclear factor κB ligand, the factor critical for osteoclast differentiation and function (56).

A role for IL-8 in stimulating bone metastasis appears at first paradoxical given the observations that IL-8 inhibits bone resorption by rat osteoclasts (55). However, human osteoclasts have been shown to secrete high constitutive levels of IL-8, which has been suggested to act as an important regulatory signal for bone, vascular, and immune cell recruitment and activation during normal and pathological bone remodeling (52). In addition, IL-8 mRNA expression is also stimulated by M-CSF, which stimulates the differentiation and proliferation of osteoclast progenitors (57). In pathological states (such as bone metastasis), the availability of IL-8 to developing osteoclasts could significantly enhance the colonization of bone by tumor cells. This intriguing idea is currently the subject of intense investigation.

In summary, the aggressive bone metastatic phenotype of IL-8 expressing MDA-MET breast cancer cells supports the possibility that IL-8 is one of the factors involved in bone metastasis. Additional investigations of the specific role of IL-8 in the metastatic phenotype of MDA-MET cells may provide critical insight into the mediators of breast cancer metastasis to bone and identify potential targets for therapeutic intervention.

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