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J Clin Invest. 1996;**98**(7):1544-1549. <https://doi.org/10.1172/JCI118947>.

Research Article

Breast cancer almost invariably metastasizes to bone in patients with advanced disease and causes local osteolysis. Much of the morbidity of advanced breast cancer is a consequence of this process. Despite the importance of the problem, little is known of the pathophysiology of local osteolysis in the skeleton or its prevention and treatment. Observations in patients with bone metastases suggest that breast cancer cells in bone express parathyroid hormone-related protein (PTHrP) more frequently than in soft tissue sites of metastasis or in the primary tumor. Thus, the role of PTHrP in the causation of breast cancer metastases in bone was examined using human breast cancer cell lines. Four of eight established human breast cancer cell lines expressed PTHrP and one of these cell lines, MDA-MB-231, was studied in detail using an *in vivo* model of osteolytic metastases. Mice inoculated with MDA-MB-231 cells developed osteolytic bone metastasis without hypercalcemia or increased plasma PTHrP concentrations. PTHrP concentrations in bone marrow plasma from femurs affected with osteolytic lesions were increased 2.5-fold over corresponding plasma PTHrP concentrations. In a separate experiment, mice were treated with either a monoclonal antibody directed against PTHrP(1-34), control IgG, or nothing before tumor inoculation with MDA-MB-231 and twice per week for 26 d. Total area of osteolytic lesions was significantly lower in mice treated with PTHrP antibodies compared with [...]

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Evidence for a Causal Role of Parathyroid Hormone–related Protein in the Pathogenesis of Human Breast Cancer–mediated Osteolysis

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Abstract

Breast cancer almost invariably metastasizes to bone in patients with advanced disease and causes local osteolysis. Much of the morbidity of advanced breast cancer is a consequence of this process. Despite the importance of the problem, little is known of the pathophysiology of local osteolysis in the skeleton or its prevention and treatment. Observations in patients with bone metastases suggest that breast cancer cells in bone express parathyroid hormone–related protein (PTHrP) more frequently than in soft tissue sites of metastasis or in the primary tumor. Thus, the role of PTHrP in the causation of breast cancer metastases in bone was examined using human breast cancer cell lines. Four of eight established human breast cancer cell lines expressed PTHrP and one of these cell lines, MDA-MB-231, was studied in detail using an *in vivo* model of osteolytic metastases. Mice inoculated with MDA-MB-231 cells developed osteolytic bone metastasis without hypercalcemia or increased plasma PTHrP concentrations. PTHrP concentrations in bone marrow plasma from femurs affected with osteolytic lesions were increased 2.5-fold over corresponding plasma PTHrP concentrations. In a separate experiment, mice were treated with either a monoclonal antibody directed against PTHrP (1-34), control IgG, or nothing before tumor inoculation with MDA-MB-231 and twice per week for 26 d. Total area of osteolytic lesions was significantly lower in mice treated with PTHrP antibodies compared with mice receiving control IgG or no treatment. Histomorphometric analysis of bone revealed decreased osteoclast number per millimeter of tumor/bone interface and increased bone area, as well as decreased tumor area, in tumor-bearing animals treated with PTHrP antibodies compared with respective controls. These results indicate that tumor-produced PTHrP can cause local bone destruction in breast cancer metastatic to

bone, even in the absence of hypercalcemia or increased circulating plasma concentrations of PTHrP. Thus, PTHrP may have an important pathogenetic role in the establishment of osteolytic bone lesions in breast cancer. Neutralizing antibodies to PTHrP may reduce the development of destructive bone lesions as well as the growth of tumor cells in bone. (*J. Clin. Invest.* 1996. 98:1544–1549.) Key words: bone metastases • hypercalcemia • parathyroid hormone–related protein • osteoclast • malignancy

Introduction

Breast cancer metastasis to bone is responsible for much of the disabling morbidity (pain, pathological fractures, hypercalcemia) in patients with advanced disease. This morbidity, a consequence of bone destruction, is due to increased osteoclast activity (1), but the mechanisms involved are poorly understood. In a majority of breast cancer patients with bone metastases, local osteolysis occurs without hypercalcemia (2), increases in nephrogenous cyclic AMP (3), or parathyroid hormone–related protein (PTHrP)¹ (4). Previous studies have suggested that production of PTHrP is more common in metastatic breast cancer cells in bone (5) than in the primary tumor (6, 7), but a role for PTHrP in the establishment and progression of osteolytic metastasis has not been tested experimentally.

In this report we show that PTHrP may be responsible for the local bone destruction occurring in patients with breast cancer, even in the absence of hypercalcemia or increased plasma PTHrP concentrations. Using an *in vivo* model of human breast cancer metastasis to bone, we demonstrate that: (a) PTHrP concentrations are increased in bone marrow plasma from bones containing metastatic tumor cells, despite the absence of increased circulating plasma PTHrP concentrations and hypercalcemia; (b) neutralizing antibodies to PTHrP significantly inhibit local osteolysis caused by metastatic human breast cancer cells; and (c) antibodies to PTHrP decrease osteolytic bone destruction and the tumor burden in bone.

Methods

Cells

The following cell lines were cultured in the respective media: RWGT2 (8), MDA-MB-231 (9), CHO-K1, and Hs578T in DMEM (Life Technologies, Inc., Grand Island, NY); BT549, ZR-75-1, T-47D,

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Received for publication 6 June 1996 and accepted in revised form 30 July 1996.

J. Clin. Invest.

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0021-9738/96/10/1544/06 \$2.00

Volume 98, Number 7, October 1996, 1544–1549

1. Abbreviations used in this paper: Ca²⁺, whole blood ionized calcium concentration; PTHrP, parathyroid hormone–related protein.

and BT483 in RPMI (JRH Biosciences, Lenexa, KS) with 10 µg/ml bovine insulin (Biofluids, Inc., Rockville, MD); MDA-MB-435s in Leibovitz L-15 (Life Technologies, Inc.); MCF-7 in IMEM (Biofluids, Inc.) with 10 µg/ml bovine insulin. All media contained 10% FCS (Hyclone, Logan, UT), 0.1% penicillin/streptomycin and nonessential amino acids (GIBCO BRL, Gaithersburg, MD). Cells were cultured in a 37°C atmosphere of 5% CO₂/95% air. MDA-MB-231 cells were provided by Dr. C.K. Osborne (University of Texas Health Science Center at San Antonio, San Antonio, TX) and all other cell lines except RWGT2 were obtained from the American Type Culture Collection (Rockville, MD). To determine PTHrP concentration in conditioned media, cell lines were simultaneously plated on 48-well plates at a cell density of 10⁴/ml and grown to confluence. Media (250 µl) were conditioned in the absence of serum for 48 h. Cells were trypsinized and counted after collection of conditioned media. Media samples were stored at -70°C until assayed for PTHrP. For each cell line, PTHrP was measured in triplicate and corrected for cell number.

For growth experiments *in vitro*, MDA-MB-231 cells were plated at a cell density of 10⁴/ml on 24-well plates. One half of the wells were treated with murine monoclonal PTHrP-(1-34) antibody (10) (10 µg/ml), described below, while the other half were similarly treated with control IgG. Three wells from each group were counted daily for 8 d.

To prepare for left cardiac inoculation, cells were trypsinized, washed twice with PBS, and resuspended in PBS to a final concentration of 10⁵ cells/100 µl.

Animals

Animal studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Female BALB/c nude mice (Audie Murphy Veteran's Administration Hospital, San Antonio, TX), 4–6 wk of age, were housed in a laminar flow isolator. Water and autoclaved mouse chow (Ralston Purina Co., St. Louis, MO) were provided *ad libitum*.

Whole blood samples for ionized calcium (Ca²⁺) determination were obtained by retroorbital puncture under anesthesia. Blood samples for PTHrP measurements were similarly obtained and collected on ice, into vacutainer tubes containing EDTA (Beckton Dickinson, Inc., Rutherford, NJ) and aprotinin (Sigma Chemical Co., St. Louis, MO), 400 KIU/ml. In the first experiment, femurs with radiologic evidence of metastases from mice inoculated via the left cardiac ventricle with MDA-MB-231 cells or femurs from control mice were harvested by flushing marrow contents with 500 µl of serum-free DMEM into iced tubes containing EDTA and aprotinin. Whole blood and marrow samples were centrifuged at 4°C for 10 min and the supernatant (bone marrow plasma) immediately frozen at -70°C until assayed for PTHrP. PTHrP concentrations in plasma and bone marrow plasma from each experiment were determined in the same assay.

Tumor inoculation into the left cardiac ventricle was performed on anesthetized mice positioned ventral side up (11). The left cardiac ventricle was punctured percutaneously using a 27-gauge needle attached to a 1-ml syringe containing suspended tumor cells. Visualization of bright red blood entering the hub of the needle in a pulsatile fashion indicated a correct position in the left cardiac ventricle. Tumor cells were inoculated slowly over 1 min.

Experimental protocols

PTHrP production in bone *in vivo*. Mice were inoculated into the left cardiac ventricle on day 0 with either MDA-MB-231 cell suspension or PBS after baseline radiographs were obtained, and Ca²⁺ and plasma PTHrP concentrations were measured (*n* = 5/group). Radiographs were taken at day 21 and at time of killing to follow the progression of osteolytic lesions. Ca²⁺ and body weight were measured on days 7, 14, 21, 24, and at time of killing, day 26. At death, blood was collected for Ca²⁺ and PTHrP measurement, bone marrow plasma was collected for PTHrP measurement and all bones and soft tissues were harvested in formalin for histologic analysis. Autopsies were performed on all mice, and those with tumors adjacent to the

heart were excluded from analysis as this indicated that part or all of the initial tumor inoculum had not entered the left cardiac ventricle.

Effects of PTHrP antibody on MDA-MB-231-induced bone metastases. Mice were divided into four treatment groups (*n* = 7/group) and inoculated with MDA-MB-231 cells into the left cardiac ventricle on day 0. Treatment, administered at a dose of 75 µg subcutaneously twice per week, starting 7 d before tumor inoculation and continued throughout the experiment, consisted of: (a) a murine monoclonal antibody directed against human PTHrP-(1-34) (PTHrP Ab; Mitsubishi, Japan) (10); (b) control IgG (IgG; Sigma Chemical Co.); (c) nothing; or (d) PTHrP Ab was given to a fourth group just before tumor inoculation and administered at the same dose and schedule for the remainder of the experiment. Radiography, Ca²⁺ and PTHrP measurement, bones and soft tissue harvest, and autopsy were performed as in the previous experiment. Results from the two antibody groups were pooled in the final analysis as all parameters measured were similar regardless of whether the antibody treatment was initiated 7 d before or on the same day as tumor inoculation.

Analytical methods

Ca²⁺ measurement. Ca²⁺ concentrations were measured using a Ciba Corning 634 ISE Ca⁺⁺/pH analyzer (Corning Medical and Scientific, Medfield, MA) and adjusted to pH 7.4 (8). Samples were run in duplicate and the mean value recorded.

PTHrP assay. PTHrP concentrations were measured in conditioned media and plasma using a two-site immunoradiometric assay (Nichols Institute, San Juan Capistrano, CA) that uses two polyclonal antibodies specific for the NH₂-terminal-(1-40) and -(60-72) portions of PTHrP and has a sensitivity of 0.3 pM (12). PTHrP concentrations were calculated from a standard curve using Prism (GraphPAD Software for Science, San Diego, CA) on an IBM-compatible computer. PTHrP concentrations in conditioned media samples were calculated from a standard curve generated by adding recombinant PTHrP-(1-86) to the specific type of medium being tested and were considered undetectable if media concentrations were < 0.3 pM before correction for cell number.

Radiographs and measurement of osteolytic lesion area. Animals were radiographed in a prone position against film (X-O mat AR; Eastman Kodak Co.) and exposed at 35 KVP for 6 s using a Cabinet X-ray System-Faxitron Series (43855A; Faxitron Corp., Buffalo Grove, IL). Films were developed using a Konica film processor. Radiographs were evaluated by three investigators in a blinded fashion. The area of osteolytic metastases was measured in both fore- and hindlimbs of all mice using an image analysis system in which radiographs were visualized using a fluorescent light box (Kaiser, Germany) and Macro TV Zoom lens 18–108 mm f2.5 (Olympus Corp., Japan) attached to a video camera (DXC-151; Sony Corp., Japan). Video images were captured using a frame grabber board (Targa+; Truevision, USA) with an IBM compatible 486/33 MHz computer. Quantitation of lesion area was performed using image analysis software (Jandel Video Analysis, Jandel Scientific, Corte Madera, CA).

Bone histology and histomorphometry. Fore- and hindlimb long bones were removed from mice at time of killing, fixed in 10% buffered formalin, decalcified in 14% EDTA, and embedded in paraffin wax. Sections were cut using a standard microtome, placed on poly-L-lysine-coated glass slides and stained with hematoxylin, eosin, orange G, and phloxine.

The following variables were measured in midsections of tibiae and femora, without knowledge of treatment groups, to assess tumor involvement: total bone area, total tumor area, and osteoclast number expressed per millimeter of tumor/bone interface. Histomorphometric analysis was performed on an OsteoMeasure System (Osteometrics, Atlanta, GA) using an IBM compatible computer.

Statistical analysis

All results are expressed as the mean ± SEM. Data were analyzed by repeated measures analysis of variance followed by Tukey-Kramer post test. *P* values of < 0.05 were considered significant.

Results

Production of PTHrP by human breast cancer cell lines in vitro. Of the eight breast cancer cell lines tested for PTHrP secretion in vitro, four produced low, but significant, amounts of PTHrP (Table I). The PTHrP concentration in media conditioned by MDA-MB-231 cells was 5.4 ± 1.0 pM/ 10^6 cells per 48 h. This was significantly less than media conditioned by a squamous carcinoma of the lung, RWGT2, established from a patient with humoral hypercalcemia (8) (21.6 ± 2.3 pM/ 10^6 cells per 48 h), but more than was secreted by Chinese hamster ovarian cells (undetectable).

MDA-MB-231 production of PTHrP in vivo. Mice inoculated with 10^5 MDA-MB-231 cells into the left cardiac ventricle developed radiographic evidence of osteolytic lesions over a period of 3 wk. Mice were killed at 4 wk after tumor inoculation. Ca^{2+} and plasma PTHrP concentrations at death were not significantly different from respective values before tumor inoculation (1.28 ± 0.05 mM vs 1.29 ± 0.03 mM for Ca^{2+} ; 1.04 ± 0.06 pM vs 1.05 ± 0.09 pM for PTHrP). In contrast, PTHrP concentrations in bone marrow plasma harvested from femurs infected with osteolytic lesions were significantly higher than corresponding plasma PTHrP concentrations (2.46 ± 0.34 pM vs 1.05 ± 0.09 pM, $P < 0.001$). Bone marrow plasma PTHrP concentrations from femurs of non-tumor-bearing mice were below the detection limit of the assay. Thus, PTHrP produced by normal bone marrow cells was not of sufficient quantity to be detected by this method.

PTHrP antibody experiments in vivo. Since the above experiment suggested that local PTHrP production by cancer cells in bone may be important in breast cancer-mediated osteolysis, the next experiment was designed to determine the role of PTHrP in the development of MDA-MB-231-mediated osteolysis. Nude mice were treated with a murine monoclonal antibody directed against PTHrP-(1-34) (10) before intra-

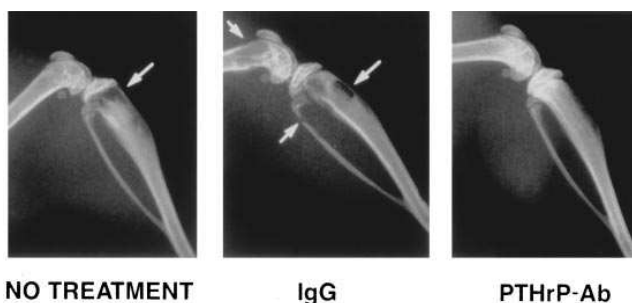


Figure 1. Radiographs of osteolytic bone lesions in hindlimbs from mice inoculated via the left cardiac ventricle from respective treatment groups. Radiographs were taken 26 d after tumor inoculation with MDA-MB-231 cells. Arrows indicate osteolytic metastases in distal femur, proximal tibia, and fibula.

cardiac inoculation of tumor cells and compared with similarly inoculated animals treated with control IgG or nothing. PTHrP Ab and control IgG were administered at a dose of $75 \mu\text{g}$ twice per week throughout the experiment. Mice were killed 26 d after tumor inoculation. One mouse each from the no-treatment group and the PTHrP Ab group were excluded from analysis as the tumor was adjacent to the heart at autopsy.

Fig. 1 illustrates representative radiographs taken 26 d after tumor inoculation. Obvious osteolytic lesions were present in mice that received no treatment or control IgG, while very few metastatic lesions were present in mice treated with the PTHrP Ab. The total area of radiographic osteolytic lesions from all long bones was quantified by a computerized image analysis system (Fig. 2). Lesion area was significantly less in mice treated with the PTHrP Ab compared with mice given no treatment or control IgG ($P < 0.001$). Values in the latter two groups were not statistically different. Representative histo-

Table I. PTHrP Concentrations in Conditioned Media from Cell Lines

Cell line	PTHrP
	pM/ 10^6 cells per 48 h
Human breast cancer	
MDA-MB-231	$5.4 \pm 1.0^*$
Hs578T	$4.6 \pm 0.5^{\ddagger}$
BT549	$4.4 \pm 1.2^{\ddagger}$
MDA-MB-435s	2.9 ± 1.2
ZR-75-1	ND
BT483	ND
MCF-7	ND
T-47D	ND
Human lung cancer	
RWGT2	$21.6 \pm 2.3^{\S}$
Other	
CHOK1	ND

Collection of conditioned media is described in Methods. Results are expressed as the mean \pm SEM. $^*P < 0.01$, $^{\ddagger}P < 0.05$, and $^{\S}P < 0.001$ compared with CHOK1. ND, not detectable; $n = 3$ wells per cell line. PTHrP concentrations were corrected for cell number.

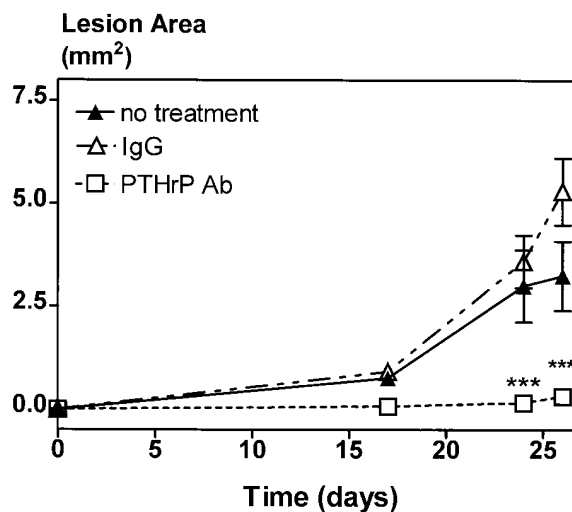


Figure 2. Osteolytic lesion area on radiographs as assessed by computerized image analysis. MDA-MB-231 cells were inoculated on day 0, and treatment (IgG or PTHrP Ab) started 7 d before tumor inoculation and continued twice per week throughout the experiment. Lesion area was measured from long bones of fore- and hindlimbs. $n = 6$ for no treatment; $n = 7$ for IgG; $n = 13$ for PTHrP Ab. Values represent the mean \pm SEM. $***P < 0.001$.

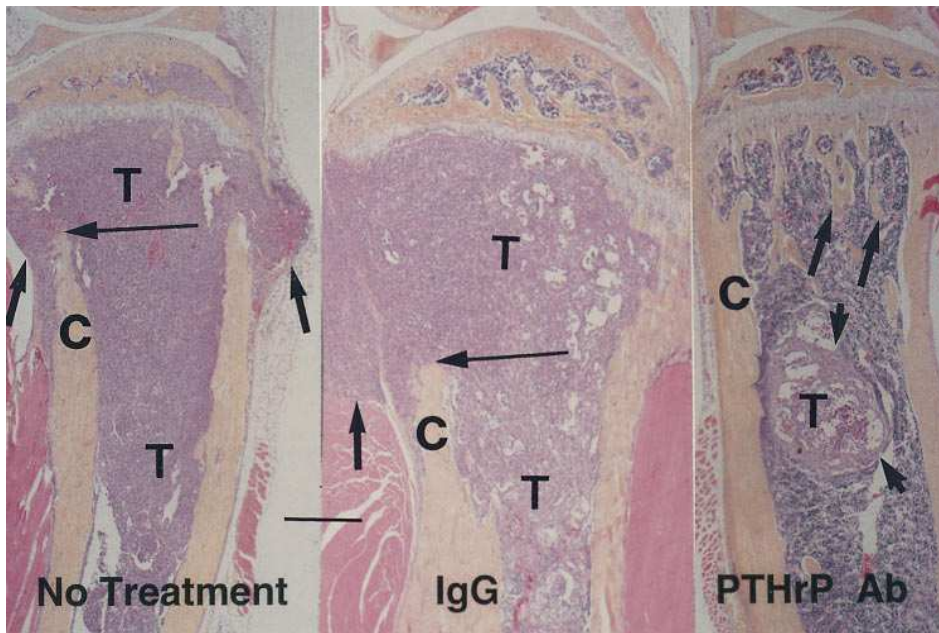


Figure 3. Effect of PTHrP Ab on MDA-MB-231 metastasis to murine tibiae 26 d after tumor inoculation. The left and middle panels illustrate sections of the tibiae from mice given no treatment or IgG. Most of the cancellous bone in the primary and secondary spongiosae has been replaced by metastatic tumor cells (T) that almost completely fill the bone marrow cavity. The cortical bone (C) has also been destroyed by osteoclasts at the proximal ends of the bones (large arrows) in response to the metastatic cancer cells. Tumor cells have spread through the cortical bone into the surrounding soft tissues (small arrows). In contrast, the right hand panel illustrates the tibia from a mouse treated with PTHrP Ab. A small deposit of metastatic tumor (T) is present within the bone marrow cavity distal to the secondary spongiosae. The bone trabeculae at the

primary and secondary spongiosae are preserved (small arrows), appear normal, and are surrounded by normal bone marrow hematopoietic tissue. The bar represents 555 μm . Hematoxylin, eosin, phloxine, and orange G staining.

logic sections through the proximal tibial metaphysis are illustrated in Fig. 3. Tumor filled the bone marrow space and destroyed both trabecular and cortical bone in mice that received no treatment or control IgG. In contrast, most of the PTHrP Ab-treated mice had intact cortical and trabecular bone and many bones had no evidence of tumor involvement. When a tumor

was present in the bone marrow space in PTHrP Ab-treated mice, it was often present as small, discrete foci within the marrow cavity and associated with little or no bone destruction.

Histomorphometric analysis of the hindlimbs from mice in all treatment groups confirmed radiographic quantitation of the osteolytic lesion area (Fig. 4). Tumor area (Fig. 4 A) and osteoclast number per millimeter of tumor/bone interface (Fig. 4 B) were significantly less in mice treated with PTHrP Ab compared with the mice that received no treatment or control IgG. Residual bone area was significantly higher in the PTHrP Ab-treated mice compared with the controls (Fig. 4 C).

Ca^{2+} concentrations remained normal in all groups for the duration of the experiment (1.26 ± 0.03 mM (PTHrP Ab), 1.28 ± 0.02 mM (no treatment), 1.28 ± 0.02 mM (IgG); $P = \text{NS}$). Body weight significantly declined in mice that received no treatment or control IgG compared with those treated with PTHrP Ab. These weight differences reached statistical significance on day 26 (21.6 ± 0.6 g (PTHrP Ab) vs 18.8 ± 0.8 g (no treatment) and 17.7 ± 0.8 g (IgG); $P < 0.05$). Additionally, no differences were evident between PTHrP Ab-treated and control mice regarding tumor cell metastases to sites other than bone. Gross and histologic examination of soft tissues revealed adrenal gland metastasis in one mouse each from the no treatment group and the PTHrP Ab group. The PTHrP Ab did not affect growth of MDA-MB-231 cells in vitro (data not shown).

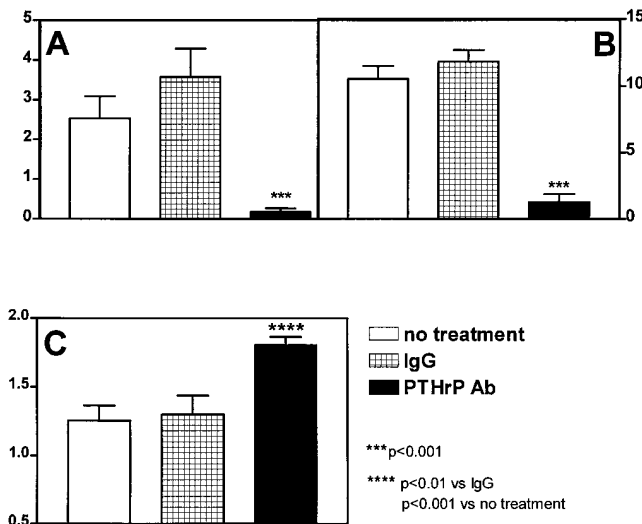


Figure 4. Histomorphometric analysis of hindlimbs from mice with MDA-MB-231 osteolytic lesions. Data represent measurements from tibiae and femurs of mice from Fig. 2 that were treated with either PTHrP Ab, IgG, or nothing. (A) Tumor area (mm^2) from MDA-MB-231 metastatic bone lesions. (B) Osteoclast number per millimeter of tumor adjacent to bone (tumor/bone interface). (C) Total bone area (mm^2) was measured in one low power magnification field ($4\times$) at the head of the tibia or femur, the site of most bone destruction. Values represent the mean \pm SEM.

Discussion

These findings demonstrate that nude mice, after inoculation with MDA-MB-231 cells into the left ventricle, develop metastatic bone disease with the same characteristic features as those seen in breast cancer patients. Tumor-bearing mice not only have osteolysis without increased plasma calcium or PTHrP concentrations, but also have enhanced production of PTHrP by tumor cells in the bone microenvironment. In our

experiments, this was evidenced by the increased PTHrP concentrations in the bone marrow plasma of affected femurs compared with blood plasma. Thus, the concentrations of PTHrP secreted *in vivo* by MDA-MB-231 cells are presumably sufficient to mediate local osteolysis, but not enough to have the systemic effects that characterize humoral hypercalcemia. Treatment with PTHrP monoclonal antibodies in this model of breast cancer-mediated osteolysis resulted in marked inhibition of the development of new osteolytic lesions and decreased osteoclast number per millimeter of tumor/bone interface, indicating that PTHrP is the critical mediator of bone destruction in this situation.

Several clinical studies in breast cancer patients indicate that plasma PTHrP concentrations are increased in ~50% of those with hypercalcemia (4, 13, 14). These data, along with the fact that one of the three tumors from which PTHrP was originally purified (15–17) was a breast carcinoma associated with humoral hypercalcemia, show that PTHrP may mediate hypercalcemia in some patients with breast cancer. In addition to this established role of PTHrP in malignancy-associated hypercalcemia, the findings presented here implicate PTHrP in the causation of breast cancer-mediated osteolysis even in the absence of hypercalcemia or increased plasma PTHrP concentrations.

One issue that arises is whether local PTHrP production by breast cancer cells in bone is a common phenomenon, and how many patients with metastatic breast cancer to bone would benefit from neutralization or inhibition of PTHrP. This issue remains to be resolved. However, our survey of PTHrP production in breast cancer cell lines demonstrates that four out of eight cell lines tested secrete significant amounts of PTHrP. These data support the clinical studies that demonstrate PTHrP expression in primary breast cancer by immunohistochemical methods to be ~50–60% (6). The clinical observations that primary breast tumors that express PTHrP are associated with the development of bone metastases (18) and that PTHrP expression by breast cancer cells in bone is greater than that of tumor cells that have metastasized to nonbone sites (5) or primary breast tumors (6, 7) are consistent with the *in vivo* data presented here.

These data and those of others show that PTHrP is frequently produced by human breast cancer cells *in vitro* and *in vivo*, and that neutralization of PTHrP may inhibit development or progression of osteolytic metastases, but they do not exclude the involvement of other mediators. Cytokines, such as tumor necrosis factor, interleukin-6, or interleukin-1, produced locally by tumor cells or normal host cells in response to the tumor, have the capacity to stimulate osteoclastic bone resorption (19). Such mediators have also been shown to modulate the end-organ effects of PTHrP (8, 20) as well as to increase its secretion from tumor cells (21). Thus, other locally produced osteolytic factors may contribute to breast cancer-induced bone destruction as well.

Immunohistochemical data from patients with metastatic breast cancer suggest that PTHrP production by tumor cells is enhanced in the bone microenvironment (5). Our data are in agreement with this concept. The capacity of breast cancer cells to express PTHrP may give them a growth advantage after they have metastasized to bone, due to the ability of PTHrP to increase osteoclastic bone resorption (22). Growth factors, such as TGF β and IGFs I and II are present within bone matrix (23) and released into the bone microenvironment as a result of osteoclastic bone resorption (24). Such

bone-derived growth factors are likely to be in high concentration and in close proximity to tumor cells in bone. TGF β enhances PTHrP expression by breast (25) and other cancers (26) and insulin-like growth factors may modulate breast cancer growth (27). Thus, once cancer cells in bone stimulate osteoclastic bone resorption, they may initiate a vicious cycle in which growth factors released from matrix enhance tumor cell growth and PTHrP production. This leads to more aggressive local bone resorption and a more favorable environment for further tumor growth and subsequent bone destruction. Since normal bone is actively remodeling, and growth factors are being released locally, PTHrP expression may be stimulated once the breast cancer cells lodge in the bone marrow stroma.

We found a marked decrease in tumor area in mice treated with PTHrP Ab, which is consistent with the notion that tumor growth is positively correlated with rates of bone resorption. In further support that neutralization of PTHrP leads to a decrease in tumor burden, tumor-bearing mice treated with PTHrP Ab maintained normal weight, whereas the controls lost a significant amount of body weight. A possible explanation is that the neutralizing antibodies to PTHrP had a direct effect on tumor growth, but this is unlikely since tumors growing in nonbone sites were not affected by PTHrP Ab, and PTHrP Ab did not affect tumor cell proliferation *in vitro*. The most likely explanation is that the antibodies neutralized the biological activity of PTHrP, thereby preventing the increase in osteoclastic bone resorption and the release of growth factors from bone that may enhance growth of the tumor cells locally. This mechanism is supported by studies demonstrating that tumor burden in bone was decreased in mice treated with bisphosphonates, selective inhibitors of osteoclastic bone resorption (28).

The observation that normal body weight was maintained in tumor-bearing mice suggests that limiting development of bone metastases with PTHrP Ab did not lead to enhanced tumor growth in other organ sites. However, the overall effect of this treatment on tumor metastasis to organ sites other than bone remains to be explored. What is clear is that neutralizing antibodies prevented destructive bone lesions and also reduced tumor mass in bone.

These data have important implications for the management of patients with breast cancer-mediated osteolysis. First, treatment directed against PTHrP, such as the antibodies used in these experiments, may prevent the development of new bone metastases and delay the progression of established metastases. Second, PTHrP expression by the primary breast tumor may be a marker for increased capacity to form bone metastases. Third, treatment with inhibitors of PTHrP or inhibitors of osteoclastic bone resorption such as bisphosphonates may be effective adjuvant therapies not only for the prevention and treatment of bone metastases, but also for reducing tumor burden by making bone a less favorable site for continued tumor growth.

Acknowledgments

The authors thank Dr. John Chirgwin for his continued support and review of the manuscript and Dr. G.D. Roodman for helpful discussions.

This study was supported by U.S. Army grant DAMD17-94-J-4231 and by the National Institutes of Health grants AR-01899 and CA-40035.

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