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PRINCIPAL INVESTIGATOR: Theresa Guise, M.D.

CONTRACTING ORGANIZATION: University of Texas
San Antonio, Texas 78229-3900

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Theresa Guise, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas
San Antonio, Texas 78229-3900

e-mail:
guise@uthscsa.edu

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Breast cancer affects the skeleton through humoral and local osteolytic mechanisms to cause the devastating complications of hypercalcemia, pain, fracture and nerve compression syndromes. PTHrP mediates hypercalcemia through its systemic effects to increase osteoclastic bone resorption as well as renal tubular calcium reabsorption in at least 50% of hypercalcemic breast cancer patients even in the presence of bone metastases. Due to this potent bone resorbing capacity, PTHrP expression in the primary tumor may aid in establishment of the bone metastases that are characteristic of patients with breast cancer. This final report summarizes the findings of the funding period. 50% of breast cancer cell lines tested secrete low, but significant amounts of PTHrP. Over-expression of PTHrP-(1-141) in the human breast cancer cell line, MDA-MB-231, increased osteolytic metastasis in a mouse model of human breast cancer metastasis to bone. Furthermore, treating mice with a neutralizing antibody to PTHrP inhibited the development of new bone metastasis and the progression of established bone metastasis caused by MDA-MB-231, a human breast cancer cell line which makes low amounts of PTHrP. Finally, of all three known isoforms of PTHrP, PTHrP-(1-139) was more efficiently secreted by breast cancer cells and caused enhanced osteolysis and hypercalcemia in vivo. These findings have important implications for the management of breast cancer and bone metastases.

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TABLE OF CONTENTS

INTRODUCTION

Breast cancer affects the skeleton through humoral and local osteolytic mechanisms to cause the devastating complications of hypercalcemia, pain, fracture and nerve compression syndromes. PTHrP is an important humoral mediator of hypercalcemia in cancer and may have physiologic roles in the lactating breast as well as in cell growth and differentiation. The role of PTHrP in the pathophysiology of breast cancer is significant for several reasons. 1) PTHrP mediates hypercalcemia through its systemic effects to increase osteoclastic bone resorption as well as renal tubular calcium reabsorption in at least 50% of hypercalcemic breast cancer patients even in the presence of bone metastases. 2) Due to this potent bone resorbing capacity, PTHrP expression in the primary tumor may aid in establishment of the bone metastases that are characteristic of patients with breast cancer. 3) Growth factors present in the bone microenvironment further enhance PTHrP expression in breast cancer cells present in bone and promote development of osteolytic lesions and tumor growth. Thus, PTHrP expression in the primary breast tumor may be a marker for the development of hypercalcemia and bone metastases. The purpose of this study was to define the role of parathyroid hormone-related protein (PTHrP) in the pathophysiology of breast cancer using animal models of breast cancer-mediated humoral hypercalcemia and osteolytic bone metastases. The following objectives were proposed: 1) to screen known breast cancer cell lines for PTHrP expression and determine if PTHrP expression was related to estrogen receptor status. 2) To determine if known human breast cancer cell lines cause hypercalcemia and if this was PTHrP-mediated. 3) To determine the role of PTHrP in the development of osteolytic metastases in breast cancer.

This final report summarizes the findings of the last 4 years of funding. In the first year, we discovered that 50% of breast cancer cell lines tested secrete low, but significant amounts of PTHrP. Over-expression of PTHrP-(1-141) in the human breast cancer cell line, MDA-MB-231, increased osteolytic metastasis in a mouse model of human breast cancer metastasis to bone. Furthermore, treating mice with a neutralizing antibody to PTHrP inhibited the development of new bone metastasis and the progression of established bone metastasis caused by MDA-MB-231, a human breast cancer cell line which makes low amounts of PTHrP. Finally, of all three known isoforms of PTHrP, 1-139, 1-141, and 1-173, PTHrP-(1-139) was more efficiently secreted by breast cancer cells. This was associated with enhanced osteolysis and hypercalcemia when the cells were studied in a mouse model of human breast cancer metastasis to bone.

In the first three years primarily one cell line, MDA-MB-231, was studied in the bone metastases model. Thus, we next focused our attention on the role of PTHrP in bone metastases caused by an estrogen receptor positive line, MCF-7. Using the nude mouse model of human breast cancer metastasis to bone, we established that MCF-7 cells cause insignificant bone metastases, while PTHrP-overexpressing MCF-7 cells avidly metastasize to bone, induced osteoclast formation, hypercalcemia which is associated with increased plasma PTHrP concentrations.

BODY

In order to define the role of PTHrP in the pathophysiology of breast cancer-associated hypercalcemia and skeletal complications in a systematic fashion, the following tasks/objectives were originally proposed.

1. **SPECIFIC AIM #1: To screen known breast cancer cell lines for PTHrP expression and secretion and to determine if PTHrP expression is related to estrogen receptor status.**

    a. Known breast cancer cell lines (both estrogen receptor positive and negative) will be grown in culture along with positive and negative controls. Media conditioned for 24 hours will be screened for PTHrP immunoreactivity by immunoradiometric assay.

    b. RNA will be isolated from above cell lines in the presence and absence of estrogen and PTHrP expression will be determined using Northern analysis.

2. **SPECIFIC AIM #2: Determine if known human breast cancer cell lines will cause humoral hypercalcemia and if this is PTHrP-mediated.**

    a. Measure standard parameters of calcium homeostasis in nude mice bearing human breast tumors.

    b. Determine that hypercalcemia observed in mice bearing PTHrP+ breast tumors is PTHrP-mediated. Two approaches will be used: i) to decrease PTHrP secretion by transfecting PTHrP + lines with PTHrP antisense ii) decrease PTHrP effects by administration of neutralizing antibody.

        1. Transfection of PTHrP antisense cDNA into breast cancer cell lines that secrete PTHrP and cause hypercalcemia in nude mice.

        2. Measurement of Ca++ in mice bearing hypercalcemic PTHrP+ breast cancer cell lines that have been transfected with PTHrP antisense cDNA.

        3. Measurement of Ca++ in mice bearing hypercalcemic PTHrP+ breast cancer cell lines that are treated with anti-PTHrP-(1-34) monoclonal antibody.

3. **SPECIFIC AIM #3: To determine the role of PTHrP in the development of osteolytic metastases in breast cancer.**

    a. Is PTHrP expression enhanced in the bone microenvironment relative to other

metastatic sites? Using an animal model of breast cancer-mediated osteolysis, PTHrP expression will be compared in bone and non-bone sites using immunohistochemistry and in situ hybridization.

b. Does expression of PTHrP in the primary tumor enhance the development and quantity of osteolytic bone metastases? Breast cancer cell line, MDA-231 will be transfected with the cDNA for human PTHrP or PTHrP-AS (antisense orientation as a control) and used in the osteolytic model.

   1. Production of stable MDA-231 clones expressing PTHrP or PTHrP-AS by calcium phosphate precipitation.

   2. Effect of MDA-231/PTHrP on development of osteolytic bone metastases will be assessed by inoculating these cells into the left ventricle of mice and determining if the quantity and size of the bone metastases differ from similarly inoculated control MDA-231/PTHrP-AS. Neutralizing antibodies will be given to attempt to block osteolysis in mice inoculated with MDA-231/PTHrP cells.

Research Accomplishment Associated with Each Task:

TASK 1. To screen known breast cancer cell lines for PTHrP expression and secretion and to determine if PTHrP expression is related to estrogen receptor status.

A panel of human breast cancer cell lines were screened for PTHrP expression and secretion as assessed by RT-PCR and 2 site immunoradiometric assay of conditioned media, respectively. The human breast cancer cell lines MDA-MB-231, Hs578T, BT549, and MDA-MB-435s (all ER-α-negative) secreted significant amounts of PTHrP while ZR-75-1, BT483, MCF-7 and T47D (all ER-α-positive) secreted no detectable PTHrP. These results are detailed in the appended manuscript (Guise et al., Journal of Clinical Investigation, 98:1544-1549, 1996; table 1). There was no effect of exogenous 17-β-estradiol or the antiestrogen tamoxifen on PTHrP production by any of the above cell lines.

TASK 2: Determine if known human breast cancer cell lines will cause humoral hypercalcemia and if this is PTHrP-mediated.

Effect of tumor-produced PTHrP on calcium balance in humoral and metastatic models: The breast cancer cell lines studied in TASK 1 were studied in mouse models of breast cancer hypercalcemia and osteolysis. When inoculated into the mammary fat pad, none of the breast cancer cell lines caused hypercalcemia. These were compared with a squamous cell carcinoma of the lung, RWGT2, which reliably causes PTHrP-mediated humoral hypercalcemia.

As part of specific aim 2a, parameters of calcium homeostasis were measured in nude mice

bearing local, nonmetastatic PTHrP-producing breast cancer cell lines and compared with nude mice bearing similar nonmetastatic PTHrP-producing tumors known to cause humoral hypercalcemia (RWGT2). In the same experiment, both tumors (MDA-MB-231 and RWGT2) were compared in the bone metastasis model. As MDA-MB-231 was one of the only breast cancer line to produce PTHrP on initial screening, it was studied in vivo in both the humoral hypercalcemia model and bone metastasis model. PTHrP production in vitro by MDA-MB-231 and RWGT2 is illustrated in table 1 of the appended manuscript (Guise et al., Journal of Clinical Investigation, 98:1544-1549, 1996).

Four weeks after local inoculation of intramuscular tumor, the mice bearing the high-PTHrP-producing RWGT2 tumors developed marked hypercalcemia while the mice bearing MDA-MB-231 remained normocalcemic. Mice inoculated with tumor cells via the left cardiac ventricle with RWGT2 had osteolytic lesions and were minimally, but significantly hypercalcemic compared with the mice bearing osteolytic lesions due to MDA-MB-231. The hypercalcemia in the metastatic RWGT2 group was mild compared with mice bearing localized RWGT2 tumors.

Hypercalcemic mice bearing local RWGT2 tumors had a significant increase in plasma PTHrP concentrations while PTHrP concentrations in normocalcemic mice bearing local MDA-MB-231 tumors did not differ from baseline. Radiographs, taken from all groups at the time of sacrifice, revealed extensive osteolytic lesions in the long bones, spine, pelvis and calvaria of mice that were inoculated with either RWGT2 or MDA-MB-231 into the left cardiac ventricle. Mice that were inoculated with local intramuscular tumors had no bone metastasis on radiographs. Histologic sections from long bones with osteolytic lesions due to MDA-MB-231 or RWGT2 revealed tumor adjacent to osteoclasts actively resorbing bone. Sections from long bones without tumor revealed no increase in osteoclastic bone resorption. Histologic sections from uninvolved calvarial bones revealed increased osteoclastic bone resorption only in the hypercalcemic group bearing local, intramuscular RWGT2 tumors. To demonstrate that PTHrP was tumor-produced in this model, local tumors as well as tumors metastatic to bone were stained by immunohistochemical technique for PTHrP. Both MDA-MB-231 and RWGT2 in muscle and in bone stained positively for PTHrP.

TASK 3: To determine the role of PTHrP in the development of osteolytic metastases in breast cancer.

MDA-MB-231 production of PTHrP in vivo: As part of specific aim 3a, PTHrP production by MDA-MB-231 cells was investigated 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 weeks. Mice were sacrificed at 4 weeks post tumor inoculation. Whole blood ionized calcium (Ca2+) and plasma PTHrP concentrations at sacrifice were not significantly different from respective values prior to tumor inoculation (1.28±0.05 mM vs. 1.29±0.03 mM for Ca2+; 1.04±0.06 pM vs. 1.05±0.09 pM for PTHrP). In contrast, PTHrP concentrations in bone marrow plasma harvested from femurs affected 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 nontumor-bearing mice were below the detection limit of the assay. Thus, PTHrP production was increased at the site of bone metastases even though the circulating PTHrP concentration were not increased. This experiment is detailed in the appended manuscript (Guise et al., Journal of Clinical Investigation, 98:1544-1549, 1996)

MDA-MB-231 overexpression of PTHrP: As part of specific aim 3b, to determine if expression of PTHrP in the primary breast tumor enhances the development and quantity of osteolytic bone metastases, MDA-MB-231 cell lines transfected with the cDNA for human PTHrP in either the sense or antisense orientation were studied in the nude mouse metastasis model. The PTHrP-overexpressing clone, MDA/PTHrP-1 secreted significantly more PTHrP than the parent MDA-MB-231 cell line. In contrast, the stable clone expressing the antisense cDNA, MDA/ PTHrP-AS, secreted less PTHrP than the parent MDA-MB-231 cell line.

To determine if PTHrP-overexpression by breast cancer cells could enhance the development of osteolytic lesions, female athymic nude mice were inoculated into the left cardiac ventricle with either parent MDA-MB-231 cells, MDA/ PTHrP-AS or high-expressing MDA/PTHrP-1. There were significantly more osteolytic lesions evident in the high-expressing MDA/ PTHrP-1 group compared with the parent MDA-MB-231 group and the MDA/PTHrP-AS group. The latter 2 groups were similar with respect to the number of bone metastases. Histologic sections of all long bones with osteolytic lesions from all groups revealed tumor adjacent to osteoclasts resorbing bone. Survival in mice bearing the high-expressing MDA/PTHrP-1 clone was significantly lower than that of either the parent MDA-MB-231 or the MDA/PTHrP-AS group. To determine if the increased number of osteolytic lesions observed in the mice bearing MDA/PTHrP-1 were a result of PTHrP to stimulate growth in MDA-MB-231 cells, cell growth in vitro was studied simultaneously in representative cell lines. No differences in cell growth were observed during the 8 day period in which the cells were in culture. The above studies are detailed in the appended manuscripts (Guise and Mundy, Endocrine Reviews, 19:18-54, 1998; Guise, Cancer, 80:1572-1580, 1997)

PTHrP antibody experiments in vivo: These experiments are detailed in the appended manuscripts (Guise et al., Journal of Clinical Investigation, 98:1544-1549, 1996; Guise, Cancer, 80:1572-1580, 1997; Guise and Mundy, Endocrine Reviews, 19:18-54, 1998). As part of specific aim 3b, to determine if expression of PTHrP in the primary breast tumor enhances the development and quantity of osteolytic bone metastases, mice inoculated with MDA-MB-231 cells were studied in the mouse metastasis model with or without treatment with PTHrP antibodies. Nude mice were treated with a murine monoclonal antibody directed against PTHrP-(1-34) prior to intracardiac 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 μg twice per week throughout the experiment. Mice were sacrificed 26 days after tumor inoculation.

Twenty-six days 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. 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. Histologic sections through the proximal tibial metaphysis demonstrated that 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 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. Tumor area and osteoclast number per mm of tumor/bone interface 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.

Ca2+ concentrations remained normal in all groups for the duration of the experiment (1.26±0.03mM (PTHrP Ab), 1.28±0.02 mM (no treatment), 1.28±0.02 mM (IgG); p=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±0.8g (no treatment) and 17.7±0.8g (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.

In a separate experiment to determine if treatment with PTHrP antibodies could delay the progression of established MDA-MB-231 bone metastasis, female athymic nude mice with radiographic osteolytic lesions 2 weeks post tumor inoculation were treated with either PTHrP-Ab or control (PBS) subcutaneously twice per week. Total radiographic lesion area was less in those animals treated with the PTHrP-Ab compared with controls ($p<0.001$).

Role of PTHrP isoforms: Since the above studies indicated that PTHrP had an important role in the development and progression of breast cancer metastasis to bone, our next aim was to investigate the role of the various isoforms of PTHrP in the pathophysiology of breast cancer metastasis to bone.

The human PTHrP gene is complex and spans approximately 15 kilobases of genomic DNA and is composed of nine exons (Southby et al., 1996). Three isoforms of PTHrP, 1-139, 1-141 and 1-173, are products of alternative splicing in humans and depend on whether exon VI is spliced to exon VII, VIII, or IX respectively. The gene is under the control of three distinct promoters and

agents which regulate PTHrP expression such as transforming growth factor β (TGFβ), glucocorticoids and epidermal growth factor act al least in part by altering the rate of gene transcription (Southby et al., 1996). However, the specific contribution of each PTHrP isoform to osteolytic metastasis caused by breast cancer or hypercalcemia is not known.

To determine the role of these isoforms in breast cancer metastasis to bone, the human breast cancer cell line MDA-MB-231 (MDA-231) was stably transfected with similar amounts of cDNAs for human prepro PTHrP-(1-139), -(1-141) or -(1-173), driven by a CMV promoter, and studied in a model of human breast cancer metastasis to bone. Stable clones expressing the cDNA for the human preproPTHrP-(1-139), -(1-141), or (1-173) secreted different amounts of PTHrP as detected by IRMA of serum-free conditioned media. All transfectants secreted significantly more PTHrP than the parental MDA-MB-231 cells. Those MDA-MB-231 cells expressing PTHrP-(1-139) consistently secreted the most PTHrP compared with the other isoforms. MDA-MB-231 cells expressing the PTHrP-(1-173) isoform secreted more PTHrP than those expressing the PTHrP -(1-141) isoform. This pattern of secretion was similar in transient transfections of 293 cells and suggests differential processing of the 1-139 isoform. In vitro growth rates were similar for all transfectants and did not differ from the parental MDA-MB-231 cells.

In vivo, bone metastases developed in all groups tested: parental MDA-MB-231, PTHrP-(1-139), -(1-141) and -(1-173). Mice bearing the MDA/PTHrP-(1-139) developed strikingly larger bone metastases which occurred much earlier than those of mice bearing the MDA/PTHrP-(1-173) or -(1-141). These differences were statistically significant as quantitated by computerized image analysis of radiographs. The total lesion area on radiographs was significantly larger in the mice bearing MDA/PTHrP-(1-139) compared with mice bearing parental MDA-MB-231, MDA/PTHrP-(1-173) and MDA/PTHrP-(1-141). The latter 3 groups did not differ significantly with regard to lesion area. Mice bearing MDA/PTHrP-(1-139) had more lesions at day 48 compared with mice in the other groups. The lesion number in mice bearing parental MDA-MB-231, MDA/PTHrP-(1-173) and MDA/PTHrP-(1-141) reached values comparable to those of the MDA/PTHrP-(1-139) group, however, this was significantly longer after tumor inoculation.

Significant hypercalcemia was evident in mice bearing MDA/PTHrP-(1-139) tumors compared with those bearing the -(1-173), -(1-141) or parental MDA-MB-231 cells. This was due to a marked increase in the plasma PTHrP concentration in the MDA/PTHrP-(1-139) group determined at sacrifice. The PTHrP concentrations were similar to those observed in humans with malignancy-associated hypercalcemia (Grill et al., 1991). Mice in all other groups remained normocalcemic throughout the experimental period. Plasma PTHrP concentrations were significantly higher in the mice bearing MDA/PTHrP-(1-173) and -(1-141) at the time of sacrifice when compared with those taken at baseline. However, these concentrations were not increased to the degree with which systemic effects of PTHrP should be observed.

In these experiments, metastasis to sites other than bone included adrenal gland, ovary, lung and liver in all groups. However, there were no significant differences in metastases to such nonbone

sites between any of the groups.

Overexpression of PTHrP into the ER-α-positive breast cancer line, MCF-7: As indicated in the initial year of this study, MCF-7 cells did not express significant amounts of PTHrP and did not cause osteolytic lesions in the mouse model. Thus, we sought to determine if overexpression of PTHrP would induce the capacity to cause osteolysis in a breast cancer cell line which was non-invasive. MCF-7 cells were transfected with the cDNA for PTHrP-(1-139) and single clones were selected. Stable clones expressing the cDNA for the human preproPTHrP-(1-139) secreted over 100-fold more PTHrP as detected by IRMA of serum-free conditioned media compared with empty vector and parental controls.

We sought to establish the role of PTHrP overexpression by the MCF-7 cell line in vivo by intracardiac injection in the nude mouse model. Mice inoculated with the MCF/PTHrP developed large bone metastases with osteolysis being evident earlier and to a greater extent than that seen with mice harboring either the parental cells or cells stably transfected with the vector control only. When quantitated by computerized image analysis of radiographs, the difference in lesion area and number were statistically significant: the increase in lesion area seen in mice harboring the vector control MCF-7 cells may be attributed to their slightly higher levels of PTHrP production relative to the parental cells. Significant hypercalcemia was evident in mice bearing the MCF/PTHrP tumors while mice bearing the MCF/EV or MCF/P tumors, which were normocalcemic. Concomitant with the hypercalcemia observed in the MCF/PTHrP bearing mice, these mice also demonstrated significantly increased plasma PTHrP concentrations at the time of sacrifice. There was no significant difference in body weight between mice bearing tumors of MCF/PTHrP, MCF/EV or MCF/P.

In these experiments, metastasis to sites other than bone included adrenal gland, ovary, lung and liver in all groups. However, there were no significant differences in metastases to such nonbone sites between any of the groups. These experiments are detailed in the appended reprint (Thomas et al., Endocrinology, 140:4451-4458, 1999).

KEY RESEARCH ACCOMPLISHMENTS

- PTHrP mediates local bone destruction (osteolysis) in the absence of hypercalcemia and increased plasma concentrations of PTHrP. Thus, PTHrP has a more universal role in malignancy as a local mediator of bone destruction, in addition to its well-known, but less common, role as a mediator of hypercalcemia.
- Neutralizing the effects of PTHrP resulted in less bone destruction as well as reduced tumor burden in a mouse bone metastases model.
- PTHrP-(1-139) isoform is produced and secreted in amounts greater than the other isoforms PTHrP-(1-141) and -(1-173). The result is increased osteoclastic bone resorption and bone metastases in a mouse model of bone metastases.
- Overexpression of PTHrP in a non-invasive breast cancer cell line which does not produce PTHrP, MCF-7, confers the capacity to form osteolytic bone metastases in a mouse model.

REPORTABLE OUTCOMES

Manuscripts (appended)

Guise TA, Yin JJ, Taylor SD, Dallas M, Boyce BF, Yoneda T, Kumaga Y, Mundy GR. Evidence for a causal role of parathyroid hormone-related protein in breast cancer mediated-osteolysis. Journal of Clinical Investigation 98:1544-1549, 1996.

Guise TA. Parathyroid hormone-related protein (PTHrP) and bone metastases. Cancer 80:1572-1580, 1997.

Mundy GR, Guise TA. Hypercalcemia of Malignancy. American Journal of Medicine 103:134-145, 1997.

Guise TA, Mundy GR. Cancer and Bone. Endocrine Reviews 19(1):18-54,1998.

Boyce BF, Yoneda T, Guise TA. Factors regulating the growth of metastatic cancer in bone. Endocrine-Related-Cancer 6(3):333-47, 1999

Thomas RJ, Guise TA, Yin JJ, Elliott J, Horwood NJ, Martin TJ, Gillespie MT. Breast cancer cells interact with osteoblasts to support osteoclast formation. Endocrinology 140(10):4451-8, 1999

Abstracts/Presentations

Guise TA, Taylor SD, Boyce BF, Mundy GR. Different tumor hypercalcemic syndromes associated with parathyroid hormone-related protein production. XIIth International conference on Calcium Regulation Hormones, Melbourne, Australia, February 1995.

Yin JJ, Taylor SD, Yoneda T, Dallas M, Boyce BF, Kumagai Y, Mundy GR, Guise TA. Evidence that parathyroid hormone-related protein (PTH-rP) causes osteolytic metastases without hypercalcemia. American Society for Bone and Mineral Research Meeting, Baltimore, MD, September, 1995. (Oral Presentation)

Yin JJ, Taylor SD, Yoneda T, Dallas M, Boyce BF, Kumagai Y, Mundy GR, Guise TA. Evidence that parathyroid hormone-related protein (PTH-rP) causes osteolytic metastases without hypercalcemia. 18th Annual San Antonio Breast Cancer Symposium, San Antonio, TX, December 1995.

GuiseTA, Yin JJ, Thomas RJ, Dallas M, Cui Y, Gillespie MT. Parathyroid hormone-related protein (PTHrP)-(1-139) isoform is efficiently secreted in vitro and enhances breast cancer metastasis to bone in vivo. American Society for Bone and Mineral Research Meeting,

Cincinnati, OH, September, 1997.

Yin JJ, Spinks TJ, Cui Y, Dallas M, Guise TA. Clonal variation in parathyroid hormone-related protein (PTHrP) secretion by a human breast cancer cell line alters severity of osteolytic metastases. American Society for Bone and Mineral Research Meeting, Cincinnati, OH, September, 1997. (Oral Presentation)

Uy HL, Mundy GR, Yin JJ, Dallas M, Park H-R, Story B, Grubbs BG, Boyce BF, Roodman GD, Guise TA. Dexamethasone inhibits bone metastasis caused by human breast cancer via several mechanisms. American Society for Bone and Mineral Research Meeting, Cincinnati, OH, September, 1997.

Guise TA, Yin JJ, Thomas RJ, Dallas M, Cui Y, Gillespie MT. Parathyroid hormone-related protein (PTHrP)-(1-139) isoform is efficiently secreted in vitro and enhances breast cancer metastasis to bone in vivo. Texas Mineralized Tissue Society Meeting, Corpus Christi, August, 1997. (Oral Presentation)

Guise TA. The role of parathyroid hormone-related protein in breast cancer metastasis to bone. Department of Defense U.S. Army Medical Research and Materiel Command Breast Cancer Research Program: An Era of Hope. Washington, D.C. November, 1997.

Thomas RJ, Guise TA, Yin JJ, Elliott J, Horwood NJ, Martin TJ, Gillespie MT. Breast cancer cells stimulate osteoblastic osteoclast differentiation factor (ODF) to support osteoclast formation. American Society for Bone and Mineral Research Meeting, December 1998, San Francisco, CA.

Thomas RJ, Guise TA, Yin JJ, Elliott J, Horwood NJ, Martin TJ, Gillespie MT. Breast cancer cells stimulate osteoblastic osteoclast differentiation factor (ODF) to support osteoclast formation. 2nd International Conference on Cancer-Induced Bone Diseases. Davos, Switzerland, March, 1999.(oral presentation)

Yin JJ, Gillespie MT, Thomas RJ, Brubbs BG, Dallas M, Cui Y, Guise TA. MCF-7 cells overexpressing parathyroid hormone-related protein (PTHrP) cause osteolytic metastases in the absence of estrogen supplementation. 81st Annual Meeting of The Endocrine Society, San Diego, CA, June, 1999. (oral presentation)

Patents and licenses applied for and issued: none

Degrees obtained that are supported by this award: none

Development of cell lines, tissue or serum repositories: Breast cancer cell lines (MCF-7 and MDA-MB-231) which overexpressed PTHrP were developed.

Informatics such as databases and animal models: A mouse model of human breast cancer metastases to bone was used for the proposed work.

Funding applied for based on work supported by this award:

FIRST Award, National Institutes of Health (NCI), "Breast cancer metastasis to bone: The role of PTHrP and TGFβ". (#R29-CA69158). Awarded for period 4/96-3/2001, annual direct cost: $70,000; total direct cost $350,000, total direct and indirect cost: $498,922. PI: TA Guise

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National Institute of Health (NCI) "Molecular mechanisms of osteoblastic bone metastases: role of endothelin-1" (R01CA81522). Awarded for period 12/99-11/04. Total direct costs: $842,582; total costs: 1,216,170. Award declined 7/99 due to overlap with P01. PI: TA Guise

Employment or research opportunities applied for and/or received on experiences/training supported by this award: see above research grants which were awarded.

CONCLUSIONS

These findings demonstrate that nude mice, following 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 approximately 50% of those with hypercalcemia (Bundred et al., 1991, 1992; Grill et al., 1991). These data, along with the fact that one of the 3 tumors from which PTHrP was originally purified (Burtis et al., 1987; Moseley et al., 1987; Strewler et al., 1987) 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 which 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 demonstrate that 5 out of 9 cell lines tested secrete significant amounts of PTHrP. These data support the clinical studies which demonstrate PTHrP expression in primary breast cancer by immunohistochemical methods to be approximately 50-60% (Southby et al., 1990; Bundred et al., 1991, 1992). The clinical observations that primary breast tumors which express PTHrP are associated with the development of bone metastases (Bundred et al., 1991, 1992) and that PTHrP expression by breast cancer cells in bone is greater than that of tumor cells which have metastasized to non-bone sites (Powell et al., 1991) or primary breast tumors (Southby et al., 1990) 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. Such mediators have also been shown to modulate the end-organ effects of PTHrP as well as to increase its secretion from tumor cells. 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 (Powell et al., 1991). 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. Growth factors, such as transforming growth factor beta (TGFβ) and insulin-like growth factors I and II are present within bone matrix (Hauschka et al., 1986) and released into the bone microenvironment as a result of osteoclastic bone resorption (Pfeilschifter et al., 1987). Such bone-derived growth factors are likely to be in high concentration and in close proximity to tumor cells in bone. TGFβ enhances PTHrP expression by breast (Yin et al., 1999) and other cancers (Southby et al., 1996) and insulin-like growth factors may modulate breast cancer growth. 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 non-bone 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 which 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 (Hortabagyi et al. 1996).

The observation that normal body weight was maintained in tumor-bearing mice suggest 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.

Overexpression of PTHrP-(1-139) in the human breast cancer cell line, MDA-MB-231, is associated with enhanced PTHrP secretion in vitro compared with other isoforms of PTHrP-(1-

173) and -(1-141). This enhanced PTHrP secretion was also evident in vivo as mice bearing the MDA/PTHrP-(1-139) tumors had increased plasma PTHrP concentrations that were significantly different from those at baseline or those at sacrifice in mice bearing parental MDA-MB-231, MDA/PTHrP-(1-173) or MDA/PTHrP-(1-141). The enhanced PTHrP secretion by MDA-MB-231 cells in vitro and in vivo correlated to increased bone destruction and hypercalcemia in a mouse model of human breast cancer metastasis to bone.

The exact role of the different isoforms of PTHrP in cancer and in normal physiology are not known. Additionally, whether any isoform predominates in malignancy is unknown. Previous work has demonstrated that all isoforms are present in the malignant cell lines BEN, COLO 16, HcCaT, MCF7, MDA-MB-231 and T-47D at the mRNA level and that expression is cell-specific with regulator-induced promoter usage (Southby et al., 1996). What is clear from the work presented here is that overexpression of the PTHrP-(1-139) isoform results in more efficient secretion of PTHrP from MDA-MB-231 cells as well as from a small number of other cell lines which have been tested such as HEK 293 cells and the human breast cancer cell line, MCF7. This efficient secretion of PTHrP results in the enhanced osteolysis and hypercalcemia observed in this mouse model of bone metastases. Whether this enhanced capacity to cause bone metastasis is an isoform-specific property rather than due to an absolute increase in PTHrP production alone cannot be determined from these studies.

Finally, overexpression of PTHrP-(1-139) in the human breast cancer cell line, MCF-7, is associated with enhanced PTHrP secretion in vitro as well as in vivo since mice bearing the MCF/PTHrP tumors had increased plasma PTHrP concentrations. The enhanced PTHrP secretion by MCF-7 cells in vitro and in vivo correlated to increased bone destruction and hypercalcemia in a mouse model of human breast cancer metastasis to bone. While parental MCF-7 cells had a low prevalence for metastasis in bone, as a result of overexpression of PTHrP, these cells avidly metastasize to bone, induced osteolysis with accompanying hypercalcemia, conditions noted in patients if breast cancers are not clinically managed.

Compiling the present data with that in the literature, a possible mechanism for the severe osteolysis induced by breast cancers is proposed. As a result of breast cancer cells establishing in the bone microenvironment, PTHrP secreted from these cells can act in a paracrine/juxtacrine manner acting on osteoblastic cells to increase expression of the recently described osteoclast differentiation factor (ODF) (Thomas et al., 1999). This favors the formation of osteoclasts, and the survival of osteoclast since ODF has also been demonstrated to limit osteoclast apoptosis. Enhancement of osteoclast numbers and their activity results in pronounced osteolysis with the subsequent release of bone-derived growth factors such as transforming growth factor β TGFβ. TGFβ is a potent stimulator of PTHrP production acting both transcriptionally and post-transcriptionally via mRNA stabilization. The demonstration that breast-cancer-cell-derived PTHrP can modulate osteolysis provides unique secreted targets to address for therapy to limit osteolysis as a result of breast cancer metastasis 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

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APPENDICES

1. Bibliography of all publications and meeting abstracts
2. The following manuscripts are appended:

Guise TA, Yin JJ, Taylor SD, Dallas M, Boyce BF, Yoneda T, Kumaga Y, Mundy GR. Evidence for a causal role of parathyroid hormone-related protein in breast cancer mediated-osteolysis. Journal of Clinical Investigation 98:1544-1549, 1996.

Guise TA. Parathyroid hormone-related protein (PTHrP) and bone metastases. Cancer 80:1572-1580, 1997.

Mundy GR, Guise TA. Hypercalcemia of Malignancy. American Journal of Medicine 103:134-145, 1997.

Guise TA, Mundy GR. Cancer and Bone. Endocrine Reviews 19(1):18-54,1998.

Boyce BF, Yoneda T, Guise TA. Factors regulating the growth of metastatic cancer in bone. Endocrine-Related-Cancer 6(3):333-47, 1999

Thomas RJ, Guise TA, Yin JJ, Elliott J, Horwood NJ, Martin TJ, Gillespie MT. Breast cancer cells interact with osteoblasts to support osteoclast formation. Endocrinology 140(10):4451-8, 1999

BIBLIOGRAPHY OF ALL PUBLICATIONS AND MEETING ABSTRACTS

Manuscripts (appended)

Guise TA, Yin JJ, Taylor SD, Dallas M, Boyce BF, Yoneda T, Kumaga Y, Mundy GR. Evidence for a causal role of parathyroid hormone-related protein in breast cancer mediated-osteolysis. Journal of Clinical Investigation 98:1544-1549, 1996.

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Yin JJ, Taylor SD, Yoneda T, Dallas M, Boyce BF, Kumagai Y, Mundy GR, Guise TA. Evidence that parathyroid hormone-related protein (PTH-rP) causes osteolytic metastases without hypercalcemia. American Society for Bone and Mineral Research Meeting, Baltimore, MD, September, 1995. (Oral Presentation)

Yin JJ, Taylor SD, Yoneda T, Dallas M, Boyce BF, Kumagai Y, Mundy GR, Guise TA. Evidence that parathyroid hormone-related protein (PTH-rP) causes osteolytic metastases without hypercalcemia. 18th Annual San Antonio Breast Cancer Symposium, San Antonio, TX, December 1995.

GuiseTA, Yin JJ, Thomas RJ, Dallas M, Cui Y, Gillespie MT. Parathyroid hormone-related protein (PTHrP)-(1-139) isoform is efficiently secreted in vitro and enhances breast cancer metastasis to bone in vivo. American Sociey for Bone and Mineral Research Meeting,

Cincinnati, OH, September, 1997.

Yin JJ, Spinks TJ, Cui Y, Dallas M, Guise TA. Clonal variation in parathyroid hormone-related protein (PTHrP) secretion by a human breast cancer cell line alters severity of osteolytic metastases. American Society for Bone and Mineral Research Meeting, Cincinnati, OH, September, 1997. (Oral Presentation)

Uy HL, Mundy GR, Yin JJ, Dallas M, Park H-R, Story B, Grubbs BG, Boyce BF, Roodman GD, Guise TA. Dexamethasone inhibits bone metastasis caused by human breast cancer via several mechanisms. American Society for Bone and Mineral Research Meeting, Cincinnati, OH, September, 1997.

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Personnel who received pay from the research efforts

Juan Juan Yin, M.D., Ph.D.
Suzanne Taylor, B.S.
Yong Cui, B.S.

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

Theresa A. Guise,* Juan Juan Yin,* Suzanne D. Taylor,* Yoshinari Kumagai,‡ Mark Dallas,* Brendan F. Boyce,§ Toshiyuki Yoneda,* and Gregory R. Mundy*

*Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284; ‡Big Bear Bio, Inc., San Mateo, California 94402; and §Department of Pathology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

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,

Address correspondence to Dr. Theresa A. Guise, Division of Endocrinology, Department of Medicine, 7703 Floyd Curl Drive, San Antonio, TX 78284-7877. Phone: 210-567-4900; FAX: 210-567-6693; E-mail: guise@uthscsa.edu

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1. *Abbreviations used in this paper:* Ca^{2+}, 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% CO_2/95% 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 10^4/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 10^4/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 10^5 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 (Ca^{2+}) 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 Ca^{2+} 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. Ca^{2+} and body weight were measured on days 7, 14, 21, 24, and at time of killing, day 26. At death, blood was collected for Ca^{2+} 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, Ca^{2+} 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

Ca^{2+} measurement. Ca^{2+} 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 NH_2-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 intracardiac 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 μg 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.

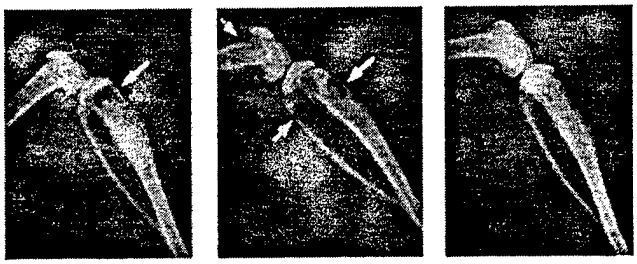

Figure 1. Radiographs of osteolytic bone lesions in hindlimbs from mice inoculated via the left cardiac ventricle from respective treatment groups. Radiographs were taken 26 d after tumor inoculation with MDA-MB-231 cells. Arrows indicate osteolytic metastases in distal femur, proximal tibia, and fibula.

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±1.0*
Hs578T	4.6±0.5‡
BT549	4.4±1.2‡
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±2.3‖
Other	
CHOK1	ND

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

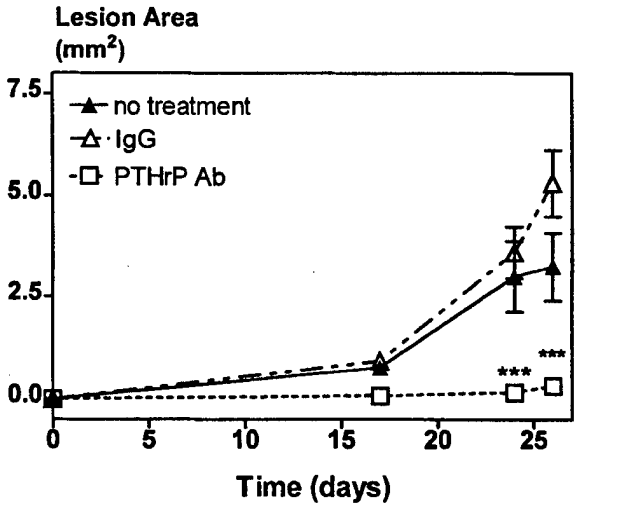

Figure 2. Osteolytic lesion area on radiographs as assessed by computerized image analysis. MDA-MB-231 cells were inoculated on day 0, and treatment (*IgG* or *PTHrP Ab*) started 7 d before tumor inoculation and continued twice per week throughout the experiment. Lesion area was measured from long bones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

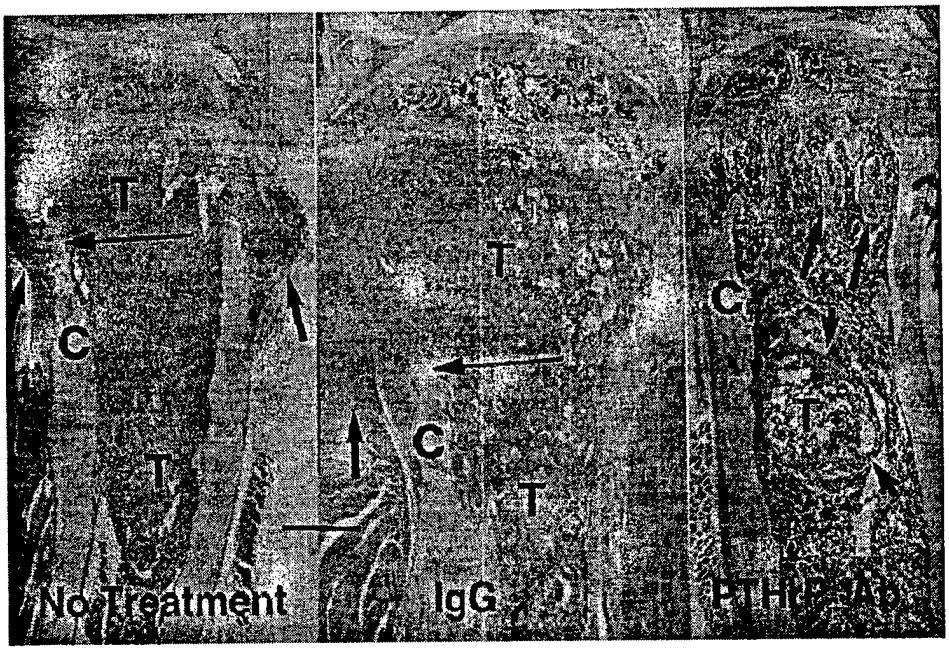

Figure 3. Effect of PTHrP Ab on MDA-MB-231 metastasis to murine tibiae 26 d after tumor inoculation. The left and middle panels illustrate sections of the tibiae from mice given no treatment or IgG. Most of the cancellous bone in the primary and secondary spongiosae has been replaced by metastatic tumor 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 = 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).

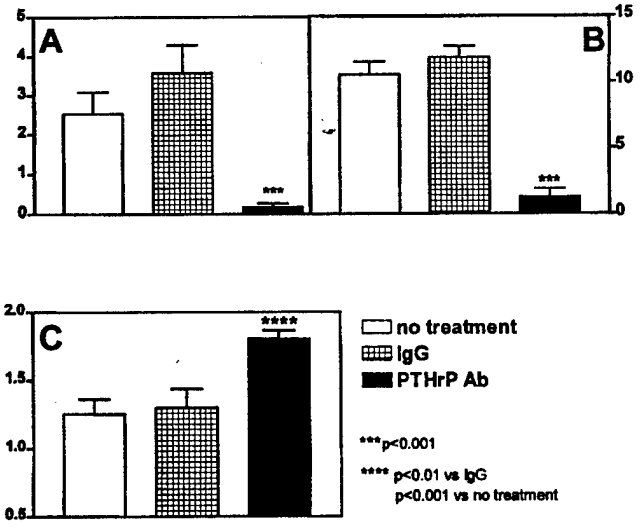

Figure 4. Histomorphometric analysis of hindlimbs from mice with MDA-MB-231 osteolytic lesions. Data represent measurements from tibiae and femurs of mice from Fig. 2 that were treated with either PTHrP Ab, IgG, or nothing. (*A*) Tumor area (mm^2) from MDA-MB-231 metastatic bone lesions. (*B*) Osteoclast 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×) at the head of the tibia or femur, the site of most bone destruction. Values represent the mean±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 TGFβ 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. TGFβ 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.

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Skeletal Complications of Malignancy

Supplement to ***Cancer***

Parathyroid Hormone–Related Protein and Bone Metastases

Theresa A. Guise, M.D.

Division of Endocrinology, Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas.

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Address for reprints: Theresa A. Guise, M.D., Division of Endocrinology, Department of Medicine, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284-7877.

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Parathyroid hormone-related protein (PTH-rP) was purified and cloned 10 years ago as a factor responsible for the hypercalcemia associated with malignancy. Clinical evidence supports another important role for PTH-rP in malignancy as a mediator of the bone destruction associated with osteolytic metastasis. Patients with PTH-rP positive breast carcinoma are more likely to develop bone metastasis. In addition, breast carcinoma metastatic to bone expresses PTH-rP in >90% of cases, compared with only 17% of metastasis to nonbone sites. These observations suggest that PTH-rP expression by breast carcinoma cells may provide a selective growth advantage in bone due to its ability to stimulate osteoclastic bone resorption. Furthermore, growth factors such as transforming growth factor-β (TGF-β), which are abundant in bone matrix, are released and activated by osteoclastic bone resorption and may enhance PTH-rP expression and tumor cell growth. To investigate the role of PTH-rP in the pathophysiology of breast carcinoma metastasis to bone, the human breast carcinoma cell line MDA-MB-231 was studied in a murine model of human breast carcinoma metastasis to bone. A series of experiments were performed in which 1) PTH-rP secretion was altered, 2) the effects of PTH-rP were neutralized, or 3) the responsiveness to TGF-β was abolished in MDA-MB-231 cells. Cultured MDA-MB-231 cells secreted low amounts of PTH-rP that increased fivefold in response to TGF-β. Tumor cells inoculated into the left cardiac ventricle of nude mice caused osteolytic metastasis similar to that observed in humans with breast carcinoma. When PTH-rP was overexpressed in the tumor cells, bone metastases were increased. MDA-MB-231 cells transfected with the cDNA for human preproPTH-rP secreted a tenfold greater amount of PTH-rP and caused significantly greater bone metastases when inoculated into the left cardiac ventricle of female nude mice compared with parental cells. In contrast, when the biologic effects of PTH-rP were neutralized or its production was suppressed, such metastases were decreased. Treatment of mice with a neutralizing monoclonal antibody to human PTH-rP resulted in a decrease in the development and progression of bone metastasis due to the parental MDA-MB-231 cells. Similar results were observed when mice were treated with dexamethasone, a potent glucocorticoid that suppresses production of PTH-rP by the MDA-MB-231 cells in vitro. The role of bone-derived TGF-β in the development and progression of bone metastasis was studied by transfecting MDA-MB-231 cells with a cDNA encoding a TGF-β type II receptor lacking a cytoplasmic domain, which acts as a dominant negative to block the cellular response to TGF-β. Stable clones expressing this mutant receptor (MDA/TβRIIΔcyt) did not increase PTH-rP secretion in response to TGF-β stimulation compared with controls of untransfected MDA-MB-231 or those transfected with the empty vector. Mice inoculated into the left cardiac ventricle with MDA/TβRIIΔcyt had fewer and smaller bone metastases as assessed radiographically and histomorphometrically compared with controls. Taken together, these data suggest that PTH-rP expression by breast carcinoma cells enhance the development and progression of breast carcinoma metastasis to bone. Furthermore, TGF-β responsiveness of breast carcinoma cells may be important for the

expression of PTH-rP in bone and the development of osteolytic bone metastasis in vivo. These interactions define a critical feedback loop between breast carcinoma cells and the bone microenvironment that may be responsible for the alacrity with which breast carcinoma grows in bone. ***Cancer* 1997;80:1572–80.**

KEYWORDS: parathyroid hormone-related protein (PTH-rP), osteolysis, bone metastasis, breast carcinoma, hypercalcemia.

Cancer metastatic to bone often causes bone destruction or osteolysis resulting in bone pain, fracture, hypercalcemia, and nerve compression syndromes. Although several tumor types, such as prostate, lung, renal cell, and thyroid carcinoma, are associated with osteolytic lesions, breast carcinoma is the most common association. A comprehensive review of >500 patients with late stage breast carcinoma revealed that 69% had bone metastasis and bone was the most common site of first distant recurrence.[1] In those patients with disease confined to the skeleton, the median survival was 24 months compared with 3 months in those patients whose first recurrence occurred in the liver. Because patients with breast carcinoma may survive several years with their bone metastases, it is important to understand the pathophysiology of this process to improve therapy and prevention. This overview will present the clinical and experimental evidence that support the role of parathyroid hormone-related protein (PTH-rP) in the pathophysiology of metastasis to bone, with specific attention to patients with breast carcinoma.

The fact that breast carcinoma is associated with significant morbidity in the skeleton was noted in 1889 when Stephen Paget observed that "in a cancer of the breast the bones suffer in a special way, which cannot be explained by any theory of embolism alone."[2] Indeed, breast carcinoma is one of a limited number of primary neoplasms that display osteotropism, an extraordinary affinity to grow in bone. Greater than 70% of women with late stage breast carcinoma have bone metastasis.[3–5] The mechanisms underlying this osteotropism are complex and involve unique characteristics of both the breast carcinoma cells and the bone to which these tumors metastasize.

Why is breast carcinoma one of the limited primary tumors to display osteotropism? Paget, during his observations of breast carcinoma in 1889, proposed the "seed and soil" hypothesis to explain this phenomenon. "When a plant goes to seed, its seeds are carried in all directions; but they can only grow if they fall on congenial soil."[2] In essence, the microenvironment of the organ to which the cancer cells metastasize may serve as a fertile soil on which the cancer cells (or seeds) may grow. Although this concept was proposed over a century ago, it remains a basic principle in the field of cancer metastasis at the current time. Thus, breast carcinoma cells possess certain properties that enable them to grow in bone and the bone microenvironment provides a fertile soil on which to grow.

Because bone is mainly comprised of a hard mineralized tissue, it is more resistant to destruction than other soft tissues. Thus, for cancer cells to grow in bone, they must possess the capacity to cause bone destruction. Histologic review of breast carcinoma metastatic to bone reveals that tumor cells are adjacent to osteoclast-resorbing bone[6–8] and indicate that breast carcinoma cells possess the capacity to stimulate osteoclastic bone resorption. Breast carcinoma cells may either induce osteoclastic differentiation of hematopoietic stem cells, activate mature osteoclasts already present in bone, or do both, through releasing soluble mediators or via cell-to-cell contact. Clinical and experimental evidence indicates that tumor-produced PTH-rP is a major candidate factor responsible for the osteoclastic bone resorption present at sites of breast carcinoma metastatic to bone.[9–11] PTH-rP is a major factor mediating malignancy-associated hypercalcemia, but recent evidence indicates that it may have an even more common role in malignancy to mediate local osteolysis even in the absence of hypercalcemia.

PTH-rP was purified from human lung carcinoma,[12] breast carcinoma,[13] and renal cell carcinoma[14] as a hypercalcemic factor simultaneously by several independent groups in 1987 and was cloned shortly thereafter.[15] PTH-rP has 70% homology to the first 13 amino acids of the N-terminal portion of parathyroid hormone (PTH),[15] binds to PTH receptors,[16] and shares similar biologic activity to PTH.[17] Specifically, it stimulates adenylate cyclase in renal and bone systems,[13,14,17–19] increases renal tubular reabsorption of calcium and osteoclastic bone resorption,[18,19] decreases renal phosphate uptake,[17,18,20] and stimulates 1α-hydroxylase.[17] PTH-rP has been demonstrated in a variety of tumor types associated with hypercalcemia including squamous, breast, and renal carcinoma.[21,22]

The hypercalcemia of malignancy syndrome was the first identified consequence of the effects of PTH-rP in cancer. In this syndrome, tumor-produced PTH-rP interacts with PTH receptors in the bone and kidney to cause hypercalcemia, osteoclast-mediated bone resorption, increased nephrogenous cyclic adenosine monophosphate, and phosphate excretion. The PTH-like properties of PTH-rP, and specifically increasing osteoclastic bone resorption and renal tubular calcium reabsorption, are responsible for the hypercalcemia. Approximately 80% of hypercalcemic patients with solid tumors have detectable or increased plasma PTH-rP concentrations.[23]

PTH-rP expression is increased by such tumor-associated products as epidermal growth factor,[24–27] insulin-like growth factor (IGF)-I[25,28] and IGF-II,[25] transforming growth factor (TGF)-α,[29] TGF-β,[27,30–32] and the *src* protooncogene[33] as well as by other hormones such as prolactin,[34] insulin,[25] and angiotensin II.[35] Glucocorticoids[25,28,36,37] and $1,25(OH)_2D_3$[24,25] decrease PTH-rP expression. An understanding of the regulation of PTH-rP expression by tumor cells is essential to study its role as a local mediator of osteolysis.

The association between PTH-rP and breast carcinoma is not a surprising one because PTH-rP is expressed in normal breast tissue and has an important role in normal breast development and physiology.[38] One of the three tumors from which PTH-rP was originally purified was a breast carcinoma from a patient with humoral hypercalcemia of malignancy.[13] PTH-rP was detected by immunohistochemical staining in 60% of 102 invasive breast tumors removed from normocalcemic women, but not in normal breast tissue.[39] Several other studies have confirmed these percentages[40–42] and one demonstrated immunoreactive PTH-rP within the cytoplasm of lobular and ductal epithelial cells in normal and fibrocystic breast tissues.[40] Furthermore, 65–92% of hypercalcemic breast carcinoma patients (with and without bone metastasis) had detectable plasma PTH-rP concentrations by radioimmunoassay similar to those documented in patients with humoral hypercalcemia of malignancy due to nonbreast tumors.[42,43] Not only is PTH-rP an important mediator of hypercalcemia in patients with breast carcinoma, it may have a significant role in the pathophysiology of breast carcinoma metastasis to bone as evidenced by the clinical studies that indicate that PTH-rP expression by primary breast carcinoma is more commonly associated with the development of bone metastasis and hypercalcemia.[43]

Breast Carcinoma Cells as the "Seed"

Although certain properties of tumor cells, such as production of proteolytic enzymes, are common to tumor cells metastasizing to any organ, such properties are insufficient to explain the propensity of breast carcinoma to metastasize to bone. Thus, breast carcinoma cells likely have additional characteristics that are specifically required for causing metastases to bone. Based on clinical and experimental studies, PTH-rP is a candidate tumor factor responsible for the osteoclastic bone resorption present at sites of breast carcinoma metastatic to bone.[9–11] Indeed, PTH-rP expression by breast carcinoma appears to enhance the ability of the breast carcinoma cell as the "seed" to grow as bone metastases due to its bone-resorbing capacity.

This notion was prompted by the observations of Powell et al., in which PTH-rP was detected by immunohistochemistry[9] and in situ hybridization[10] in 92% of breast carcinoma metastases in bone compared with only 17% of similar metastases to nonbone sites. These data are supported by the work of Bundred et al., who found positive immunohistochemical staining for PTH-rP in 56% of 155 primary breast tumors from normocalcemic women. In this study, PTH-rP expression was positively correlated to the development of bone metastases and hypercalcemic episodes.[42] In addition, PTH-rP expression was detected by reverse transcriptase polymerase chain reaction in 37 of 38 primary breast carcinomas, and subsequent development of bone metastases was associated with a higher PTH-rP expression.[11] There have been no consistent correlations between PTH-rP expression in the primary breast tumor and standard prognostic factors, recurrence, or survival. The only significant and consistent correlations have been between PTH-rP positivity and the development of bone metastases and hypercalcemia.

These clinical observations have been extended by experimental studies using a mouse model of bone metastases[44,45] in which inoculation of a human breast carcinoma cell line, MDA-MB-231,[46] into the left cardiac ventricle reliably causes osteolytic metastases, usually in the absence of hypercalcemia or increased plasma PTH-rP concentrations. MDA-MB-231 cells produce low amounts of PTH-rP in vitro and when the cells were engineered to overexpress PTH-rP, an increase in the number of osteolytic metastases was observed.[47] Specifically, MDA-MB-231 were transfected with 1) the cDNA for human preproPTH-rP driven by a cytomegalovirus promoter to produce PTH-rP-overexpressing clones and 2) the PTH-rP cDNA in the antisense orientation to depress PTH-rP secretion. Stable clones expressing high and low concentrations of PTH-rP were selected for study in vivo. Mice inoculated into the left ventricle with the high expressing clone, MDA/PTH-rP-1, had 3-fold

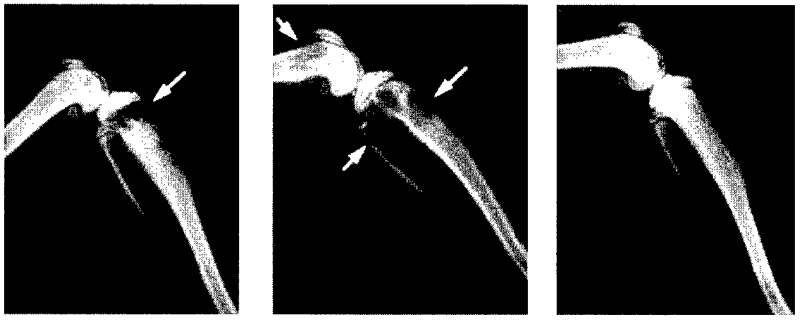

FIGURE 1. Representative radiographs of osteolytic bone lesions in hindlimbs from mice inoculated via the left cardiac ventricle from respective treatment groups. Radiographs were taken 26 days after tumor inoculation with MDA-MB-231 cells. Arrows indicate osteolytic metastases in distal femur, proximal tibia, and fibula. IgG: immunoglobulin G; PTH-rP-Ab: parathyroid hormone-related protein antibody. Reproduced from *J Clin Invest* 1996;98:1544–9 by copyright permission of the American Society for Clinical Investigation.

more osteolytic bone lesions radiographically at 3 weeks compared with mice inoculated with the low expressing antisense clone, MDA/PTHrP-AS, or with the untransfected MDA-MB-231 cells. Mice in the MDA/PTHrP-1 group became mildly hypercalcemic compared with mice in the MDA/PTHrP-AS or parental MDA-MB-231 group, but neither had detectable plasma PTH-rP concentrations. Survival was significantly less in the mice bearing MDA/PTHrP-1 compared with the antisense or parental group. These data demonstrate that osteolytic lesions were significantly enhanced by PTH-rP overexpression in MDA-MB-231 cells and that the effects were local because plasma PTH-rP concentrations were undetectable.

In contrast, when mice were treated with monoclonal antibodies directed against the 1-34 region of PTH-rP prior to inoculation with parental MDA-MB-231 cells, the number and size of observed osteolytic lesions were dramatically less than observed in similar animals treated with control immunoglobulin G or no treatment (Figs. 1 and 2). Histomorphometric analysis of long bones from tumor-bearing mice revealed significantly fewer osteoclasts per mm of the tumor-bone interface in mice treated with the PTH-rP antibody compared with the controls (Figures 3 and 4). This is predictable because neutralizing the effects of PTH-rP should decrease osteoclastic bone resorption. However, the intriguing aspect of this was that tumor burden in bone, assessed histomorphometrically, was significantly less in the PTH-rP antibody-treated mice inoculated with MDA-MB-231 tumor cells compared with similar tumor-bearing mice treated with control. Thus, neutralizing the effects of PTH-rP not only decreases osteoclastic bone resorption but also tumor

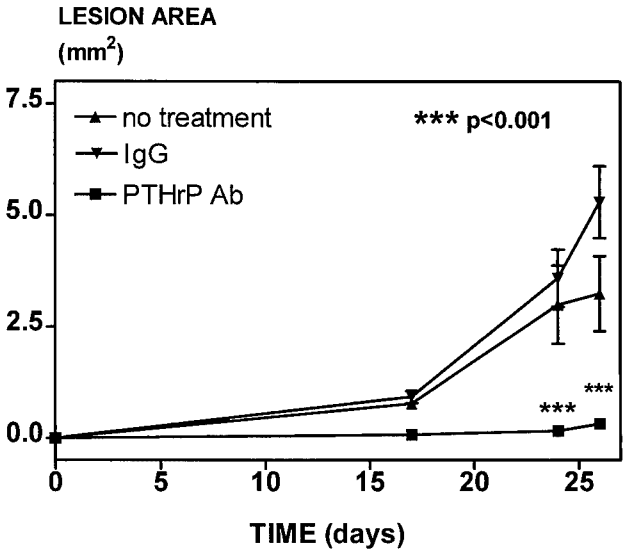

FIGURE 2. Osteolytic lesion area on radiographs as assessed by computerized image analysis. MDA-MB-231 cells were inoculated on Day 0, and representative treatment (immunoglobulin G [IgG] or parathyroid hormone-related protein antibody [PTH-rP Ab]) started 7 days prior to tumor inoculation and continued twice per week throughout the experiment. Lesion area was measured from long bones of fore- and hindlimbs (N = 7 mice/group). All values represent the mean ± standard error of the mean. Standard error bars are not visualized at some of the time points because they fall within the size of the symbol. Reproduced from *J Clin Invest* 1996;98:1544–9 by copyright permission of the American Society for Clinical Investigation.

burden in bone. In a separate experiment, mice with established osteolytic metastases due to MDA-MB-231, treated with the PTH-rP antibody, had a decrease in the rate of progression of metastases when compared with mice that received a control injection.[48,49]

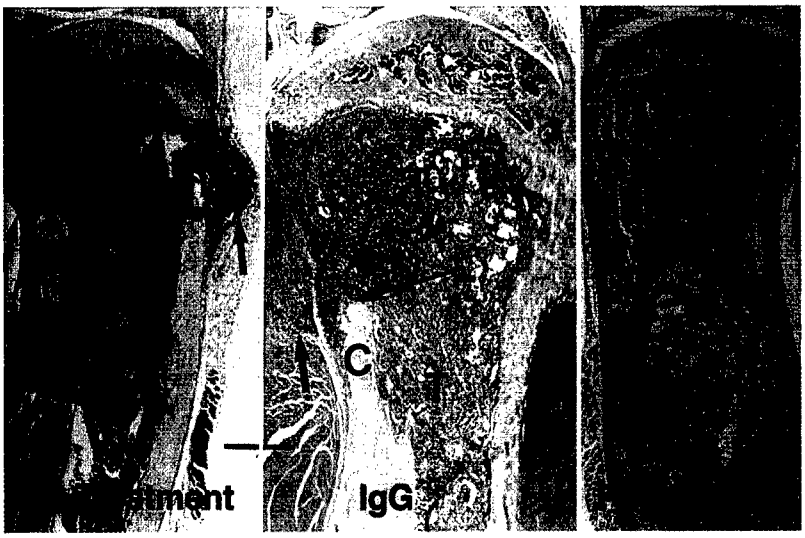

FIGURE 3. Effect of parathyroid hormone-related protein (PTH-rP) antibody (PTH-rP Ab) on MDA-MB-231 cell metastasis to murine tibiae. MDA-MB-231 breast carcinoma cells were injected into the left ventricle of nude mice that were sacrificed 26 days later. Mice also were given 75 μg of PTH-rP Ab or immunoglobulin G (IgG) twice weekly or else received no concomitant treatment. The left and middle panels illustrate representative sections of the tibiae from mice who received no treatment or treatment with IgG. Most of the cancellous bone in the primary and secondary spongiosae had been replaced by metastatic tumor cells (T) which almost completely filled the bone marrow cavity. The cortical bone (C) was also destroyed by osteoclasts at the proximal ends of the bones (large arrows) in response to the metastatic cancer cells. Tumor cells spread through the cortical bone into the surrounding soft tissues (small arrows). In contrast, the right panel illustrates the tibia from a representative mouse injected with tumor cells and given PTH-rP Ab. A relatively small deposit of metastatic tumor (T) was present within the bone marrow cavity distal to the secondary spongiosae. The bone trabeculae at the primary and secondary spongiosae were preserved (small arrows), appeared normal, and were surrounded by normal bone marrow hematopoietic tissue. The bar represents 555 μm (Hematoxylin, eosin, phloxine, and orange G staining). Reproduced from *J Clin Invest* 1996;98:1544–9 by copyright permission of the American Society for Clinical Investigation.

Because neutralizing the effects of PTH-rP had a significant impact on reducing tumor burden in bone, one would predict that decreasing the production of PTH-rP by tumor cells would have similar effects. Glucocorticoids inhibit PTH-rP secretion in vitro,[36,37] but whether this can prevent bone metastases mediated by PTH-rP is unknown. To determine whether glucocorticoid treatment would reduce tumor-produced PTH-rP and bone metastasis, the effect of dexamethasone on PTH-rP production and the development and progression of bone metastasis caused by MDA-MB-231 was studied. Dexamethasone significantly decreased PTH-rP mRNA and protein production, in a dose-dependent manner, by MDA-MB-231 cells. Placebo-treated mice inoculated with MDA-MB-231 into the left cardiac ventricle developed significantly more and larger bone metastases, as assessed by computerized image analysis of radiographs, compared with similarly inoculated mice treated with slow-release dexamethasone pellets (2.7 mg/kg/day). Histomorphometric analysis confirmed these data. In contrast, dexamethasone had no effect on tumor size of MDA-MB-231 cells inoculated intramuscularly. PTH-rP concentrations in bone marrow plasma from femora with osteolytic lesions were significantly higher in placebo-treated mice compared with dexamethasone-treated mice. These data suggest that dexamethasone in large doses can effectively reduce tumor burden in bone by inhibiting PTH-rP expression. The adverse metabolic, immunologic, and musculoskeletal effects of high dose glucocorticoids unfortunately preclude their use for the treatment of bone metastases in humans. However, these data serve as proof of concept to support the local role of PTH-rP in mediating breast carcinoma-induced osteolysis.

Taken together, the above clinical and experimen-

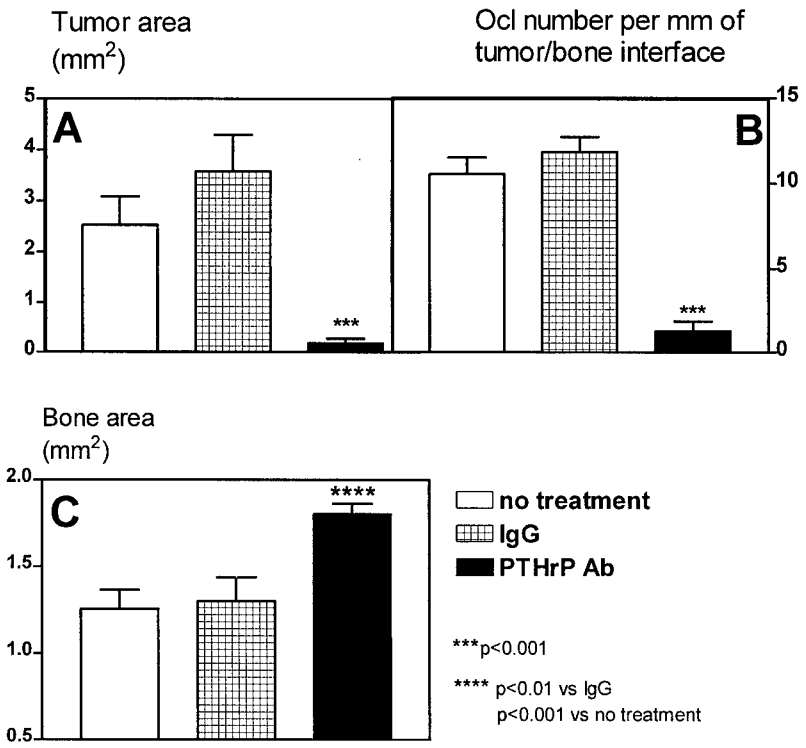

FIGURE 4. Histomorphometric analysis of hindlimbs from tumor-bearing mice with MDA-MB-231 metastatic bone lesions. Data represent measurements from tibiae and femora of mice from Figure 3 that were treated with either parathyroid hormone-related protein antibody (PTH-rP Ab), immunoglobulin G (IgG) or nothing. A) Tumor area (mm^2) from MDA-MB-231 metastatic bone lesions. B) Osteoclast number per mm of tumor adjacent to bone (tumor/bone interface). Ocl: osteoclast. C) Total bone area (mm^2) was measured in one low-power magnification field (X4) at the head of the tibia or femur, the site of most bone destruction. All values represent the mean ± the standard error of the mean. Reproduced from *J Clin Invest* 1996;98:1544–9 by copyright permission of the American Society for Clinical Investigation.

tal evidence strongly suggest that PTH-rP expression by breast carcinoma cells is important for the development and progression of breast carcinoma metastases in bone.

Bone Microenvironment as the "Soil"

The clinical data demonstrating PTH-rP expression by >90% of breast carcinoma metastasis to bone[9] compared with 17% of tumor from nonbone sites or 50% of primary breast carcinoma[39] suggest not only that PTH-rP positive primary tumors are more likely to metastasize to bone but that the bone microenvironment may enhance PTH-rP expression as well. Bone is unique among metastatic target tissues because it undergoes continual remodeling under the influence of systemic hormones and local bone-derived growth factors. Mineralized bone matrix is a repository for growth factors, of which TGF-β and IGF-II comprise the majority.[50] Such growth factors are released from the bone matrix as a result of normal osteoclastic bone resorption,[51] a part of the normal remodeling process necessary for maintenance of the structural integrity of bone. The hematopoietic stem cells in the bone marrow can differentiate into bone-resorbing osteoclasts. Other cells in the bone marrow, stromal and immune cells in particular, produce cytokines and growth factors that may potentiate tumor cell growth or expression of osteolytic factors. Thus, once breast carcinoma cells arrest in bone, the high concentrations of growth factors and cytokines in the bone microenvironment provide a fertile soil on which the cells can grow. Such host cytokines also may enhance osteoclastic bone resorption stimulated by tumor-produced factors such as PTH-rP.[52] Furthermore, when the tumor cells stimulate osteoclastic bone resorption, this bone microenvironment is even more enriched with bone-derived growth factors that enhance survival of the tumor. Finally, bone-derived TGF-β may have an important role as a chemoattractant for breast carcinoma cells.

A large body of evidence to support the concept that bone is a fertile soil, further enriched by the process of osteoclastic bone resorption, has accumulated in studies using bisphosphonates in the treatment of bone metastases. It already is clear from clinical studies that the use of bisphosphonates, potent inhibitors of bone resorption, significantly reduces skeletal morbidity in humans with advanced breast carcinoma.[53–59] Bisphosphonates also have been shown to decrease the number of bone metastases as well as tumor burden in animal models.[45,60] Thus, by decreasing osteoclastic bone resorption, the bone microenvironment is a less fertile soil for the growth of tumor.

TGF-β, which is present in high concentrations in the bone microenvironment and expressed by some breast carcinomas[61] and carcinoma-associated stromal cells,[62] has been shown to enhance secretion of and stabilize the mRNA for PTH-rP in a renal cell carcinoma,[30] a squamous cell carcinoma,[31,32] and a human breast carcinoma, MDA-MB-231.[47] In fact, of the known growth factors present in the mineralized bone matrix other than TGF-β, such as IGF-I and IGF-II, fibroblast growth factor 1 and 2, bone morphogenetic protein, and platelet-derived growth factor, only TGF-β has been shown to significantly stimulate PTH-rP secretion from the human breast carcinoma cell line, MDA-MB-231.[47] The fact that TGF-β is abundant in bone[50] and can enhance PTH-rP expression by cancer cells makes it an important candidate factor in the establishment and progression of breast carcinoma metastases to bone. TGF-β is a member of a large superfamily of proteins that are important regulators of bone cell activity.[63] Five isoforms of TGF-β exist and appear to control cell proliferation and differentiation in many human cell types.[64] The prototype of these isoforms, TGF-β1, is highly expressed by differentiated osteoblasts and osteoclasts, stored in bone matrix, and released in active form during osteoclastic bone resorption.[50]

Further evidence for the role of bone-derived TGF-β in the development and progression of breast carcinoma metastasis to bone has been demonstrated in the same in vivo animal model of osteolysis described earlier. Because TGF-β increases PTH-rP expression by MDA-MB-231 cells in vitro, this cell line was transfected with a cDNA encoding a TGF-β type II receptor lacking a cytoplasmic domain (TβRIIΔcyt).[65] This receptor binds TGF-β, but because it cannot phosphorylate the type I receptor, signal transduction is not initiated and it acts in a dominant-negative fashion to block the biologic effects of TGF-β.[66] Stable clones expressing TβRIIΔcyt did not increase PTH-rP secretion in response to TGF-β stimulation compared with controls of untransfected MDA-MB-231 cells or those transfected with the empty vector. Receptor expression in stable clones was demonstrated by crosslinking to ^{125}I-labeled TGF-β1. Growth rates were similar for MDA/TβRIIΔcyt, MDA/pcDNA3, and MDA-231 clones in vitro as well as in vivo. Mice inoculated into the left cardiac ventricle with MDA-MB-231 cells expressing TβRIIΔcyt (MDA/TβRIIΔcyt) had fewer osteolytic lesions as well as a smaller area of osteolytic lesions by radiography and histomorphometry compared with the controls of parental cells or those transfected with the empty vector (MDA/pcDNA3).[67] Osteoclast number and tumor area in bone were significantly less in the mice bearing MDA/TβRIIΔcyt compared with controls and were similar to the results observed when MDA-MB-231 tumor-bearing mice were treated with PTH-rP antibody. Survival was significantly longer in mice inoculated with MDA/TβRIIΔcyt compared with those inoculated with MDA/pcDNA3 or parental MDA-MB-231 cells. The data indicate that TGF-β responsiveness of the human breast carcinoma cells, MDA-MB-231, is important for the expression of PTH-rP in bone and the development of osteolytic bone metastasis in vivo.

Taken together, the clinical and experimental data provide strong evidence that PTH-rP expression by breast carcinoma cells enhance the development and progression of breast carcinoma metastasis to bone. Furthermore, TGF-β responsiveness of breast carcinoma cells may be important for the expression of PTH-rP in bone and the development of osteolytic bone metastasis in vivo. These interactions define a critical feedback loop between breast carcinoma cells and the bone microenvironment that may be responsible for the alacrity with which breast carcinoma grows in bone. Disruption of this feedback loop by neutralizing the effects of PTH-rP, decreasing its production, or blocking the biologic effects of TGF-β on tumor cells may significantly decrease the development and progression of breast carcinoma metastasis to bone.

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Hypercalcemia of Malignancy

Gregory R. Mundy, MD, Theresa A. Guise, MD, *San Antonio, Texas*

The hypercalcemia of malignancy is a topic that has undergone major changes in the past decade. Before that time, there was considerable controversy over the relative roles of different mediators, on the difficulties in differential diagnosis from the other nonparathyroid causes of hypercalcemia and from primary hyperparathyroidism, and debate over the place of various medical therapies. Some of these issues have now been resolved, or at least clarified, but this has led to newer questions that face clinicians treating patients with this common complication of the most common malignancies. This review focuses on some of these current issues.

PATHOPHYSIOLOGY OF HYPERCALCEMIA OF MALIGNANCY

The major cause of hypercalcemia is increased bone resorption, but there is also almost always a concomitant impairment of the renal mechanisms normally responsible for clearing the increased calcium load from the extracellular fluid. This is usually attributable to an increase in renal tubular calcium reabsorption and is caused by the parathyroid hormone-related protein (PTHrP) in many patients with solid tumors. The increase in renal tubular calcium reabsorption also occurs in patients with myeloma and breast cancer, where circulating PTHrP is often not an important factor.[1] In these latter patients, the cause of increased renal tubular calcium reabsorption is unknown. Volume depletion, which impairs glomerular filtration and leads to increased sodium and calcium reabsorption in the proximal convoluted tubules, may be partly responsible for calcium reabsorption when the patient first presents. However, increased renal tubular calcium reabsorption persists even after volume repletion.[1] In patients with multiple myeloma, hypercalcemia usually occurs in the setting of a fixed impairment of glomerular filtration independent of volume.

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From the Department of Medicine/Endocrinology, University of Texas Health Science Center, San Antonio, Texas.
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Requests for reprints should be addressed to Gregroy R. Mundy, MD, Department of Medicine/Endocrinology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284-7877.
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Although increased renal tubular calcium reabsorption is important in the pathophysiology of hypercalcemia, it is rarely the sole cause for hypercalcemia. The primary cause is increased osteoclastic bone resorption, as drugs that inhibit osteoclast activity such as bisphosphonates, gallium nitrate, or plicamycin (mithramycin) successfully lower the serum calcium concentration in most patients. The increase in osteoclastic bone resorption is due to mediators produced either by tumor cells or by host cells. The major mediator in solid tumors is PTHrP, but other mediators are also likely to be involved.

PTHrP was purified from human lung cancer,[2] breast cancer,[3] and renal cell carcinoma[4] simultaneously by several independent groups almost 50 years after Fuller Albright first proposed that the syndrome of hypercalcemia in a patient with renal carcinoma and a solitary bone metastasis was due to ectopic production of a hormone with PTH-like effects.[5] It is evident that PTHrP, and not PTH, is a major mediator of humoral hypercalcemia of malignancy, although three cases of authentic tumor-produced PTH have been reported.[6–8] PTHrP has 70% homology to the first 13 amino acids of the amino-terminal portion of PTH,[9] binds to PTH/PTHrP receptors,[10] and shares similar biologic activity to PTH.[11] Specifically, it stimulates adenylate cyclase in renal and bone systems,[12] increases renal tubular reabsorption of calcium and osteoclastic bone resorption,[12] decreases renal phosphate uptake,[11] and stimulates 1α-hydroxylase.[11] PTHrP has been found in a variety of tumor types associated with hypercalcemia including squamous, breast, and renal carcinoma.[13,14]

The human PTHrP gene, cloned shortly after its purification,[9] is much larger and more complex than the human PTH gene. It spans approximately 15 kilobases of genomic DNA and has 9 exons and 3 promoters. Three PTHrP isoforms of 139, 141, or 173 amino acids as well as multiple PTHrP messenger RNA species exist.[15] Like PTH and other endocrine peptides, PTHrP undergoes endoproteolytic post-translational processing that results in several secretory forms: (1) an amino-terminal PTHrP-(1–36), (2) a mid-region species that begins at amino acid 38 that has an undefined carboxyl terminus,[16,17] and (3) a carboxy-terminal species that is recognized by an antibody directed against the 109–138 region.[18,19] The carboxy-terminal species is cleared by the kidney, similar to PTH, as increased concentrations of

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this fragment are present in patients with chronic renal failure. In fact, the plasma carboxy-terminal PTHrP concentration increases as the glomerular filtration rate decreases.[19] Although PTHrP mediates its calcemic effects through the classic PTH/PTHrP receptor, there is accumulating evidence for a separate PTHrP receptor.[20] Whether PTHrP mediates any of its effects through this receptor is unknown. The classic PTH/PTHrP receptor belongs to the family of G protein-coupled receptors, some of which include receptors for calcitonin and secretin.[21] The PTH/PTHrP receptor is expressed in a large variety of fetal and adult tissues and activates at least two second messenger systems, adenyl cyclase and phospholipase C.[22]

PTHrP has been detected in normal as well as malignant tissue.[13,14] The widespread expression of PTHrP and the PTH/PTHrP receptor in normal tissue suggested that the protein had functions other than those of a hypercalcemic factor. In addition to the PTH-like effects, emerging work testifies to the fact that PTHrP has an integral role in varied aspects of normal physiology. Such a topic is beyond the scope of this review, but suffice it to say that PTHrP appears to be important in (1) the regulation of cartilage differentiation and bone formation through endochondral ossification[23,24]; (2) growth and differentiation of skin,[25] mammary gland,[26] and pancreatic islets[27]; (3) transepithelial calcium transport in the distal nephron, mammary epithelia, and the placenta[28,29]; (4) relaxation of smooth muscle in uterus, bladder, arteries, stomach, and ileum[30]; and (5) host immune function.[31-33] These potential normal physiologic functions of PTHrP have been reviewed extensively elsewhere.[34]

Biochemical features of humoral hypercalcemia of malignancy (HHM) share some similarities to those seen in primary hyperparathyroidism (1°HPT), as is expected, because PTH and PTHrP bind to a common receptor. Specifically, in addition to the hypercalcemia associated with malignancy, hypophosphatemia can occur (depending on the patient's renal function) as well as hypercalciuria, hyperphosphaturia, and increased excretion of nephrogenous cyclic AMP.[35] Plasma intact PTH concentrations are suppressed in this syndrome except in rare cases of ectopic PTH production or concomitant primary hyperparathyroidism. Plasma $1,25(OH)_2D_3$ concentrations are generally low except in certain hematologic malignancies when the humoral mediator is $1,25(OH)_2D_3$. Approximately 80% of hypercalcemic patients with solid tumors have detectable or increased plasma PTHrP concentrations,[18] whereas such concentrations are low or undetectable in the plasma of healthy persons.[36] Other tumor-produced factors, such as interleukin (IL)-1α, IL-6, transforming growth factor (TGF)-α, and tumor necrosis factor (TNF)-α, have been demonstrated to stimulate osteoclastic bone resorption in vitro and in vivo and to cause hypercalcemia when administered to rodents.[37-41] Thus, these factors may be implicated in the pathophysiology of the hypercalcemia in the absence of PTHrP. Furthermore, as discussed later, many of these factors may potentiate the end-organ effects of PTHrP, and subsequently, the hypercalcemia.

Figure 1 shows the heterogeneity of patterns of calcium fluxes in patients with hypercalcemia of malignancy. In patients with solid tumors shown on the right, there is increased bone resorption and increased renal tubular calcium reabsorption. In patients with (HHM) (upper right panel), there is increased nephrogenous cyclic AMP and increased plasma PTHrP. In patients with breast cancer (lower right panel), the same pattern of abnormalities is present, but there is frequently neither an increase in plasma PTHrP nor in nephrogenous cyclic AMP. In these latter patients, it is likely that PTHrP produced locally in the bone microenvironment is a major causative factor, as evidenced by recent human and rodent studies. Immunohistochemical studies demonstrate that human breast cancer metastatic to bone expresses PTHrP in more than 90% of cases, whereas only 17% of metastasis to nonbone sites express PTHrP.[42] Furthermore, in a mouse model of human breast cancer osteolysis, PTHrP production was increased in the bone marrow from tumor-bearing femurs, although simultaneous plasma PTHrP concentrations were normal.[43] In patients with multiple myeloma (upper left panel), there is an increase in bone resorption and impaired glomerular filtration. In some patients with lymphomas (lower left panel), there is increased bone resorption and increased gut absorption of calcium associated with 1,25 dihydroxyvitamin D production by the tumor tissue. It is probable, however, that the majority of patients with the lymphomas have the clinical syndrome of HHM associated with increased PTHrP production (upper right panel).

CLASSIFICATION OF HYPERCALCEMIA OF MALIGNANCY

Hypercalcemia of malignancy occurs in several different settings. These settings include HHM, where a systemic mediator produced by tumor cells, now identified as PTHrP, is responsible for hypercalcemia. A second setting is hypercalcemia due to extensive localized osteolysis caused by tumor cells, such as occurs in patients with metastatic breast cancer and multiple myeloma. This has been the standard method for classifying hypercalcemia of malignancy for 20 years, and will be followed here.

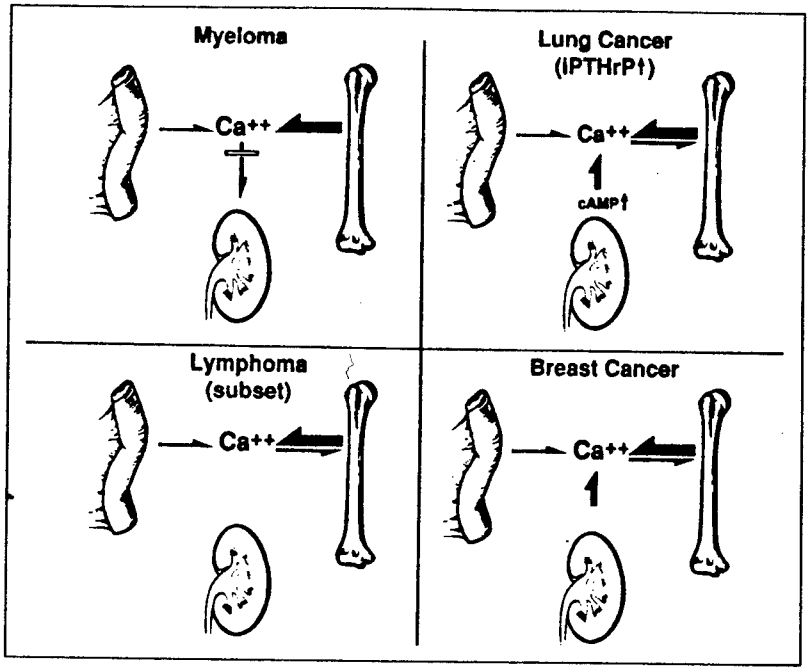

Figure 1. The pathophysiology of hypercalcemia in different types of malignancy. Upper left panel represents myeloma, where hypercalcemia is associated with an increase in bone resorption, impairment in bone formation, and decreased glomerular filtration. Upper right panel shows situations in patients with HHM, where there is increase in bone resorption and decrease in bone formation associated with increased renal tubular calcium reabsorption. PTHrP is overproduced by tumors with this syndrome, and patients have increased circulating PTHrP and nephrogenous cyclic AMP. Lower right panel shows the situation in patients with breast cancer. There is also increase in bone resorption with decreased bone formation associated with increased renal tubular calcium reabsorption. Many patients with hypercalcemia due to breast cancer do not have increases in circulating PTHrP or in nephrogenous cyclic AMP, but local production of PTHrP in the bone microenvironment is likely responsible for osteolysis and ultimate hypercalcemia. Lower left panel represents the occasional patient with increased production of 1,25 dihydroxyvitamin D, increased calcium absorption from the gut, and increased bone turnover. These patients represent a small subset of all patients with hypercalcemia due to lymphoma. Most probably have a form of HHM.

However, on the basis of current information, it is probably more accurate to think of these situations as a continual spectrum rather than splitting them into discrete groups. It is true that the pathophysiology of hypercalcemia is very different in patients with, for example, islet cell carcinoma of the pancreas with no bone metastases at one end of this spectrum, and multiple myeloma associated with extensive local bone destruction adjacent to the tumor cells at the other end of the spectrum. However, between these two extremes are hypercalcemic patients with squamous cell carcinomas where hypercalcemia may occur with some, but not extensive, osteolytic bone metastases, and hypercalcemic patients with advanced breast carcinoma, where hypercalcemia usually occurs in the presence of extensive osteolytic bone destruction. On the basis of our current understanding, separating hypercalcemia into subcategories on the assumption that the underlying mechanisms are distinct is not entirely satisfactory because the mediators may be identical. For example, HHM is characterized by hypercalcemia, increased plasma PTHrP, and increased nephrogenous cyclic AMP, and PTHrP is the major mediator. However, in some cases of local osteolysis, PTHrP is also likely responsible, although the other features of HHM may not be present.

MEDIATORS INVOLVED IN HYPERCALCEMIA OF MALIGNANCY

Solid Tumors Without Extensive Bone Metastases

This is the HHM syndrome. In these patients, typically patients with squamous cell carcinoma of the lung, head, and neck, patients with adenocarcinomas of the kidney, lung, pancreas, or ovary, the major mediator is PTHrP, and the syndrome is characterized by hypercalcemia in the presence of increased plasma PTHrP and increased nephrogenous cyclic AMP concentrations. There is a fair correlation with plasma PTHrP and hypercalcemia in these patients, and in animal models of this syndrome, hypercalcemia has been reversed by treatment with neutralizing antibodies to PTHrP.[44]

However, it is likely that PTHrP alone does not account for all of the clinical features of the HHM

TABLE I
Differences Between the Syndromes of HHM and Primary Hyperparathyroidism

	HHM	Primary Hyperparathyroidism
Bone resorption	↑↑	↑
Bone formation	↓↓	↑
Gut absorption of calcium	↓	↑
Serum 1,25 dihydroxyvitamin D_3	↓	↑
Tubular reabsorption of HCO_3	↑	↓
Serum chloride	↓	↑
Plasma PTHrP	↑	NI
Plasma PTH	↓	↑
Nephrogenous cyclic AMP	↑	↑
Plasma phosphate	↓	↓

NI = not increased; ↓ = decreased; ↑ = increased.

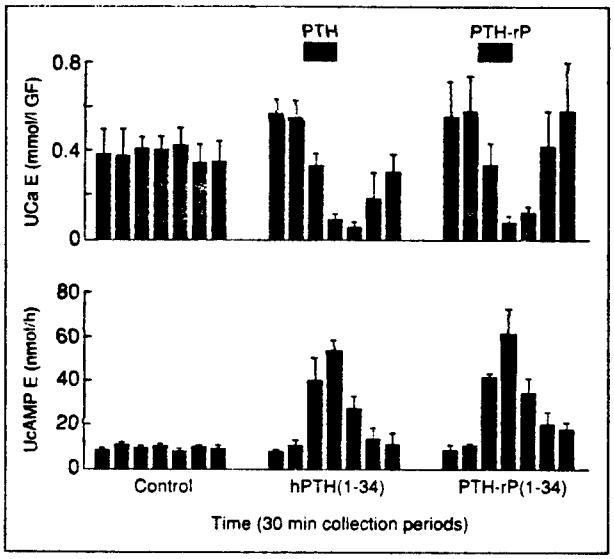

Figure 2. PTH and PTHrP appear to have identical effects on bone resorption and renal tubular calcium (UCa) reabsorption, presumably mediated through the PTH/PTHrP receptor. Differences between the syndrome of HHM and primary hyperparathyroidism are likely accounted for by other mechanisms independent of PTH/PTHrP. This figure shows the effects of PTH and PTHrP on renal tubular calcium reabsorption.[12]

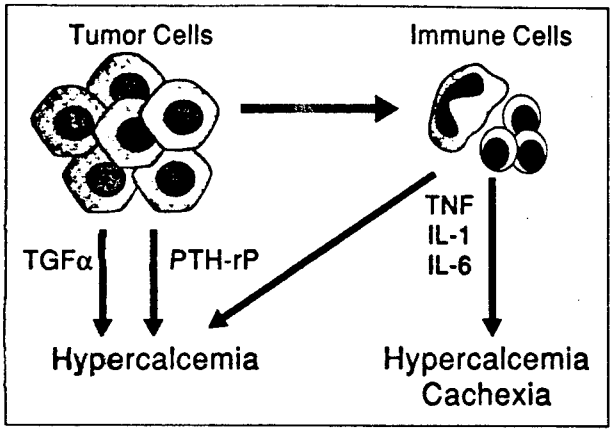

Figure 3. Concept for multiple paraneoplastic syndromes associated with hypercalcemia of malignancy mediated by PTHrP. Many solid tumors produce PTHrP. There is also frequently overproduction of cytokines such as IL-1α, TGF-α, IL-6, TNF by either tumor cells or host cells. These may be responsible for other paraneoplastic syndromes associated with hypercalcemia such as leukocytosis and cachexia (see text for details).

syndrome. There are clear differences between the syndromes of HHM and primary hyperparathyroidism, although the major mediators in both are factors that act identically on the PTH/PTHrP receptor in bone and kidney (**Table I, Figure 2**). It is clear that short-term infusions of both PTH and PTHrP cause identical effects on serum calcium, serum 1,25 dihydroxyvitamin D, and renal tubular reabsorption of calcium.[45] In most patients with malignancy, there is a decrease in calcium absorption from the gut, decreased serum 1,25 dihydroxyvitamin D concentrations, a decrease in bone formation,[46] and a decrease in serum chloride and metabolic alkalosis. This is the opposite to what is seen in patients with primary hyperparathyroidism (Table I). One explanation is that the effects of tumor-produced PTHrP on bone, kidney, and gut in these patients are influenced by other factors that are also produced because of the tumor such as IL-1α, TGF-α, TNF-α, and IL-6 (**Figure 3**). These factors all influence osteoclastic bone resorption and have been shown to increase plasma calcium when produced in excess. Specifically, IL-1α, TGF-α, TNF-α, and IL-6 potentiate the effects of PTHrP on osteoclast activity and calcium homeostasis,[47-50] although some oppose its effects on osteoblast function.[51,52] Overproduction of these factors may explain at least some of the distinctive features that are seen in patients with HHM. Other hypotheses for the discrepancies between HHM and 1°HPT include differences between the pulsatile secretion of PTH[53] and the presumed continuous secretion of PTHrP, suppression of bone formation, and 1α-hydroxylase activity by biologically active PTHrP fragments, or hypercalcemia per se.[54] Despite our growing knowledge of hypercalcemia, the reasons for these differences will only be adequately elucidated by future studies in humans.

There are other paraneoplastic syndromes that often occur in patients with HHM indicating that the entire clinical picture may be the result of a constellation of factors (Figure 3). One of these is leukocytosis and another is cachexia. HHM is often associated with leukocytosis, particularly in patients with squamous cell carcinomas.[55] This leukocytosis cannot be ascribed to obvious explanations such as infection, and recently has been linked to factors that are produced both by the tumor and by host immune cells. These include colony-stimulating factors as well as cytokines such as IL-6, TNF, and IL-1. Yoneda et al[55] have shown definitive evidence for production of some of these mediators by host im-

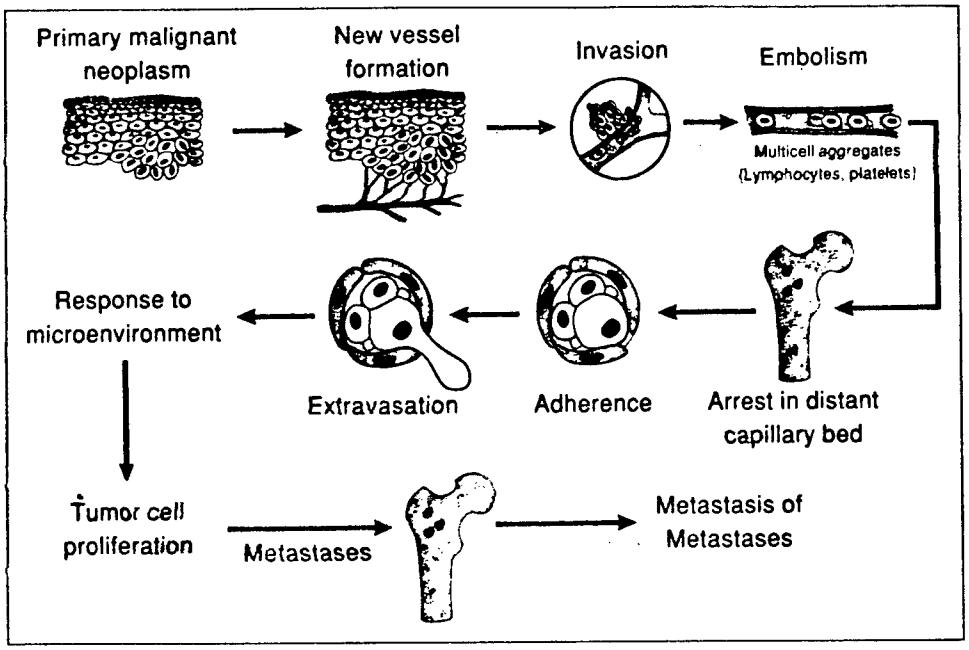

Figure 4. Multiple steps involved in tumor cell metastasis from a primary site to the skeleton. Each of these steps represents a potential therapeutic target for the development of drugs to reverse or prevent metastatic bone disease.

mune cells as well as by tumor cells. In one particular example, conditioned media harvested from human squamous cell carcinoma cells stimulated TNF production by cells in the monocyte lineage in vitro, and the hypercalcemia in tumor-bearing nude mice was alleviated by removal of the immune cells in the spleen by splenectomy, as well as by antibodies to TNF and to IL-6.[55]

Solid Tumors With Extensive Bone Metastases

The most obvious example of this type of hypercalcemia of malignancy occurs in patients with breast cancer. In this situation, hypercalcemia usually occurs late in the disease course when patients have extensive metastases at multiple organ sites, including most notably the skeleton. Hypercalcemia almost never occurs in the absence of extensive osteolytic bone metastases. The multiple steps involved in the metastasis of tumor cells from the primary site to the secondary site in bone are all important forerunners of the onset of hypercalcemia (**Figure 4**). It was formerly thought that the last of these steps, namely the bone destruction associated with metastatic cancer, was mediated directly by the tumor cells, but it is now recognized that the intervention of osteoclasts is more important, from careful observations of bone surfaces using scanning electron microscopy.[56] Histologic sections of breast cancer metastatic to bone reveal tumor adjacent to osteoclasts resorbing bone.[57] Unexpectedly, it now appears that the mediator in at least some of these cases is PTHrP, the peptide that has been associated with HHM.[42,43] It should be noted that many of these patients do not have increased plasma PTHrP or increased nephrogenous cyclic AMP, indicating that absence of these latter parameters does not mean PTHrP is unimportant in the cause of hypercalcemia. In one example in tumor-bearing nude mice, neutralizing antibodies to PTHrP have been shown to prevent the development of the osteolytic process, often the primary cause of hypercalcemia.[43] In the case of breast cancer tumor cells produce increased amounts of PTHrP in the bone microenvironment. There is a marked difference between expression of PTHrP in the primary breast cancer site or at soft tissue organ sites other than bone.[42] The most likely explanation for this is that bone provides a fertile environment for the growth of the tumor cells and also enhances the production of PTHrP in this microenvironment. One of the mechanisms by which this may occur is the production of PTHrP by the tumor cells in response to TGF-β, which is abundant in bone matrix and released from bone in an active form when bone is resorbing.[58] TGF-β increases secretion of PTHrP from tumor cells[59] and dominant-negative blockade of the type II TGF-β receptor in human breast cancer cells results in fewer bone metastasis in a mouse model.[60] A paracrine loop is initiated in which PTHrP stimulates osteoclastic bone resorption and TGF-β, released from resorbing bone, stimulates further tumor cell production of PTHrP and more osteoclastic bone resorption. However, it is also likely that there are other mechanisms for bone destruction associated with metastatic tumor.

TABLE II

Causes of Hypercalcemia

Primary hyperparathyroidism
Malignant disease
Hyperthyroidism
Immobilization
Vitamin D intoxication
Vitamin A intoxication
Familial hypocalciuric hypercalcemia (FHH)
Diuretic phase of acute renal failure
Chronic renal failure
Thiazide diuretics
Sarcoidosis and other granulomatous diseases
Milk-alkali syndrome
Addison's disease
Paget's disease

TABLE III

***Relative* Frequency of Causes of Hypercalcemia[63]**

	Number of Patients	Percentage of Cases
Primary hyperparathyroidism	111	54
Malignant disease	72	25
Lung	25	35
Breast	18	25
Hematologic (myeloma 5, lymphoma 4)	10	14
Head and neck	4	8
Renal	2	3
Prostate	2	3
Unknown primary	5	7
Others (gastrointestinal 4)	8	8

These may involve production by the tumor cells of mediators such as TGF-α and IL-1α, and production by host immune cells of cytokines such as IL-6, IL-1, and TNF-α in response to the presence of the tumor. It is probable that the combination of these factors acting in concert is responsible for the destructive bone lesions.

Multiple Myeloma

Myeloma is a disease that is almost invariably associated with destructive bone lesions, either in the form of diffuse osteopenia or localized osteolytic deposits throughout the skeleton.[61] In myeloma, bone destruction occurs as a consequence of the production of mediators by the myeloma cells such as IL-6, IL-1 and TNF-β.[39] For some years, there has been considerable controversy as to the nature of the mediator responsible for bone destruction in myeloma, and this has still not been identified definitively. Possibly all of these mediators are important. What is important in myeloma is that there is a marked increase in osteoclastic bone resorption, usually without manifestations of increased bone formation.[62] This is in contrast to breast cancer, where although the bone lesions are mainly destructive, there is usually a slight increase in bone formation and an increase in alkaline phosphatase and radionuclide uptake at sites of osteolytic deposits associated with increased osteoblast activity. This is less so in myeloma where there is a marked inhibition of osteoblast function. The mechanism responsible for this uncoupling of bone formation from bone resorption is not known, but is the subject of intense study.

As with solid tumors that cause destructive bone lesions and hypercalcemia, hypercalcemia is also frequent in myeloma, occurring in about 30% of all patients sometime during the course of the disease. The therapeutic approach is directed both at the malignant cells and at the cells that are responsible for destructive bone resorption and is discussed in more detail in a later section.

DIFFERENTIAL DIAGNOSIS OF HYPERCALCEMIA OF MALIGNANCY

Before 1987, the differential diagnosis of hypercalcemia, although obvious in many cases, required some skill. Although there are many causes for hypercalcemia, 90% of patients have either primary hyperparathyroidism or an underlying malignancy that is responsible (**Tables II, III**). As a consequence, the differential diagnosis in most patients lies between primary hyperparathyroidism and hypercalcemia of malignancy. Before 1990, the radioimmunoassay for PTH was widely used, but was far from ideal. The majority of patients with primary hyperparathyroidism had increased plasma immunoreactive PTH and in those patients who had an immunoreactive PTH concentration that was greater than 30% above normal in the presence of normal renal function, the diagnosis of primary hyperparathyroidism could be made with confidence. However, there were many patients with hypercalcemia in whom the immunoreactive PTH was not in this clear diagnostic range, and these are patients who presented diagnostic problems because many of them still had primary hyperparathyroidism. Moreover, in patients with nonparathyroid malignant disease and hypercalcemia, immunoreactive PTH could be in the normal range, slightly increased, or even suppressed. When renal failure was superimposed on the clinical picture, the plasma immunoreactive PTH concentration was even more difficult to interpret, as the kidney is responsible for clearance of some of the fragments of PTH. The most immunoreactive fragments are those that are least biologically active. To compound this problem, there was differential clearance of these fragments in the presence of renal failure.

As a consequence, clinicians developed many other subtle ways to distinguish between hypercalcemia of malignancy and primary hyperparathyroid-

ism where the cause was not obvious and the plasma immunoreactive PTH concentration did not allow a diagnosis to be made with confidence. These included careful consideration of points in the history and physical examination such as obvious presence of malignancy, age and sex of the patient, and duration of hypercalcemia. Primary hyperparathyroidism is most frequent in elderly women,[63] although it can occur at any age. Because the hypercalcemia of malignancy is rarely occult, when hypercalcemia occurs in any patient who is asymptomatic and apparently without malignant disease, primary hyperparathyroidism is the most likely diagnosis. In addition, if review of chart records for measurements of serum calcium, now made routinely with the autoanalyzer, shows a previous measurement that was either normal or clearly increased, this is useful information in the differential diagnosis (**Figure 5**). If a patient is mildly hypercalcemic for more than 6 months, the diagnosis of primary hyperparathyroidism or some other nonmalignant cause of hypercalcemia becomes much more likely. In contrast, if hypercalcemia is of recent onset, then malignancy is more likely as the cause. Other measurements were also helpful, including measurement of serum chloride. In patients with primary hyperparathyroidism, serum chloride is usually greater than 103 mmol/L, particularly if the patient has normal renal function, because of the effects of PTH on renal bicarbonate wasting to cause a mild form of renal tubular acidosis. In contrast, in patients with hypercalcemia of malignancy, the serum chloride is usually <100 mmol/L. Although a number of years ago a chloride/phosphorus ratio was advocated by some, this is not helpful in the majority of patients. Other measurements sometimes used in the differential diagnosis were urinary calcium excretion, urinary or nephrogenous cyclic AMP, radiologic evaluation for subperiosteal resorption of fingers, bone scans, measurement of serum 1,25 dihydroxyvitamin D, and even in some cases more complicated tests such as bone biopsy and histomorphometry, or steroid suppression tests.

Fortunately, these tests are not now needed in the vast majority of patients. The entire picture for the differential diagnosis of hypercalcemia has changed with the advent of the immunoradiometric assay (IRMA) for parathyroid hormone. In this technique, antibodies directed at two sites on the PTH molecule are used and cross-reactivity with inactive PTH fragments is reduced.[64] These assays measure intact PTH and do not detect inactive fragments, and clearly improve discrimination between patients with primary hyperparathyroidism and patients with hypercalcemia of malignancy. For those patients with nonparathyroid causes of hypercalcemia, PTH should be

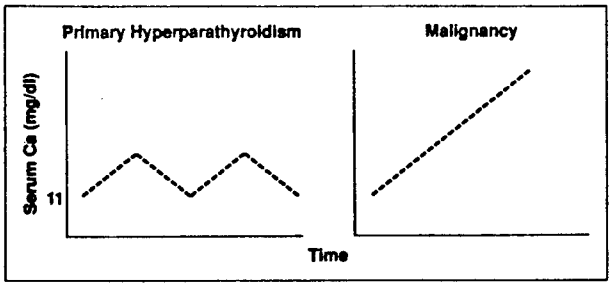

Figure 5. Differences in the natural history of hypercalcemia in patients with primary hyperparathyroidism and in patients with malignancy. In patients with primary hyperparathyroidism, serum calcium may oscillate just above the normal range for many years. In contrast, in patients with hypercalcemia of malignancy, the tumor is usually untreatable and the hypercalcemia progresses steadily as tumor bulk increases. This has important practical consequences, particular for the differential diagnosis of hypercalcemia and in the treatment of hypercalcemia. Hypercalcemia is steadily progressive in patients with malignancy, but may be present for years in patients with primary hyperparathyroidism, and this is a helpful point in the differential diagnosis if it is discovered that hypercalcemia has been present for more than 6 months. In patients with malignancy in whom the serum calcium has been lowered to the normal range by inhibitors of bone resorption, prevention of further episodes of hypercalcemia will require maintenance treatment with agents that inhibit bone resorption such as the bisphosphonates.

suppressed because of the presence of hypercalcemia. In the absence of renal failure, the immunoradiometric PTH assay is now critical in the differential diagnosis of hypercalcemia. However, there are three caveats. First, there are a few well-described patients with hypercalcemia of malignancy where PTH is the mediator.[6-8] In these patients of course, IRMAs indicate increased plasma PTH in the absence of primary hyperparathyroidism. The second caveat are those patients with malignancy who have coexistent primary hyperparathyroidism. It should be remembered that both primary hyperparathyroidism and cancer are common, and can occur in the same individual. There are numerous case reports of primary hyperparathyroidism occurring in patients with a nonparathyroid malignancy. This should be considered in all patients with increased or inappropriately normal plasma PTH concentration whether or not they have malignant disease. If a hypercalcemic patient has an increased plasma PTH concentration in the absence of renal failure, it is far more likely that the cause is concomitant primary hyperparathyroidism than an ectopic source of parathyroid hormone.

The IRMA for PTH is thus very helpful in the diagnosis of hypercalcemia of malignancy, probably even more helpful than measurement of PTHrP by radioimmunoassay or IRMA. The plasma PTH is suppressed in the vast majority of patients with the hypercalcemia of malignancy. Suppression of PTH in the presence of hypercalcemia should exclude primary hyperparathyroidism as the cause, although there are occasional patients with primary hyperpar-

athyroidism in whom PTH is not increased or inappropriately normal, even when the PTH IRMA assay is the diagnostic test. The mediator produced by the parathyroid glands in these patients is unknown.

In summary then, there are a number of points that favor a diagnosis of malignancy as the cause of hypercalcemia. In addition to suppressed plasma PTH, these include recent onset of hypercalcemia, the presence of other features of malignancy such as weight loss, anemia and in many patients with hypercalcemia impaired renal function, a serum chloride <100 mmol/L in the presence of normal renal function, serum 1,25 dihydroxyvitamin D measurements that are normal or suppressed, and bone histomorphometry where performed that shows an increase in bone resorption with impaired bone formation.

For those situations where hypercalcemia occurs in a patient with a known malignancy, it is also worth considering the type of malignancy that is present (Table III). Hypercalcemia often occurs in association with some malignancies but rarely with others. For example, hypercalcemia is common in patients with advanced carcinoma of the breast, but rare in patients with carcinoma of the breast without bone metastases. It is common in patients with squamous cell carcinoma of the lung, but rare in patients with small cell carcinoma of the lung. Hypercalcemia occurs frequently in myeloma, and occasionally in patients with T-cell lymphoma, Hodgkin's disease, or B-cell lymphomas. It is rare in patients with carcinoma of the uterus or carcinoma of the colon. On the other hand, it is common in patients with pancreatic islet cell tumors and particularly VIPomas (neoplasms of vasoactive intestinal peptide secreting cells) and cholangiocarcinomas. It is very common in squamous cell carcinomas at almost any site. It also occurs occasionally in patients with adenocarcinomas of the kidney, ovary, pancreas, and lung. Hypercalcemia may be due to an occult malignancy, but this is uncommon. In the great majority of patients with hypercalcemia of malignancy, the malignancy is obvious, the disease is widespread, and the patient has metastases in multiple organs. These patients are usually in the last few months of life.[65] It is rare for a patient with the hypercalcemia of malignancy to live more than 6 months, and many of them are dead in 1 to 3 months.

TREATMENT OF HYPERCALCEMIA OF MALIGNANCY

Before 1990, medical therapy of hypercalcemia of malignancy was based on treatment of the underlying malignancy where possible, fluid replacement with normal saline, and the use of agents such as plicamycin (mithramycin), calcitonin, glucocorticoids, or phosphate. The choice of drugs has changed considerably with the approval by the Food and Drug Administration (FDA) of pamidronate (Aredia) in the past 5 years. From that time, pamidronate has become the mainstay of treatment for the hypercalcemia of malignancy. This agent is safe, nontoxic, and efficacious in >95% of patients. It has been used extensively worldwide for treatment of hypercalcemia of malignancy with outstanding success. Pamidronate, administered as a single 24-hour infusion normalized serum calcium concentrations in 30% of patients who received 30 mg, 61% of patients who received 60 mg, and 100% of patients who received 90 mg.[66] Successful therapy with bisphosphonates is associated with an increase in the plasma PTH and $1,25(OH)_2D_3$ concentrations as well as a decrease in the biochemical markers of bone resorption.[66-68] Clinical studies of pamidronate treatment in patients with hypercalcemia of malignancy indicate that the calcium-lowering response to bisphosphonates positively correlates with the presence of bone metastases[69,70] and negatively correlates with plasma PTHrP concentrations.[69-71] This is presumably due to the effects of PTHrP to increase renal tubular reabsorption of calcium, which are not blocked by bisphosphonates. As a consequence of the efficacy of the bisphosphonates, the need for alternative agents such as plicamycin or gallium nitrate has essentially disappeared. Pamidronate should be delivered as an intravenous infusion over 4 to 24 hours. Clinical studies using a 90-mg infusion of pamidronate over 4 hours, indicate that the mean time to achieve normocalcemia is approximately 4 days and the mean duration of normocalcemia is 28 days.[72] Similarly, intravenous pamidronate, 60 mg every 2 weeks, maintained normocalcemia in a majority of patients with malignancy-associated hypercalcemia.[73] An effective method for achieving more rapid reduction of the serum calcium is to use the combination of calcitonin and pamidronate.[74] Calcitonin acts rapidly to lower the serum calcium although usually its effects are only transient. Although escape from calcitonin therapy may occur within 48 hours, by that time pamidronate is beginning to exert its maximal effects. Calcitonin can be administered either intramuscularly or subcutaneously every 12 hours in doses of 200 to 400 MRC units.

The rationale for the use of normal saline at the time of initial treatment is that volume depletion impairs glomerular filtration and leads to increased sodium and calcium reabsorption in the proximal convoluted tubules. Therapy with normal saline not only corrects volume deficits but also decreases linked sodium and calcium reabsorption in the proximal tubules. Normal saline should be administered rela-

tively vigorously as it will have the capacity not only to replete volume, but also to decrease calcium reabsorption in the proximal renal tubule. However, occasional patients may become hypernatremic because of the relative resistance to antidiuretic hormone that occurs in hypercalcemia, combined with an impairment in the thirst mechanism if they are confused or obtunded. If hypernatremia occurs, fluid administration should be continued with hypotonic fluids.

In occasional patients with severe impairment of renal function, it may be advisable to avoid some inhibitors of bone resorption such as the bisphosphonates, plicamycin, or gallium nitrate, where the agents may either be nephrotoxic or accumulate in the presence of renal failure. Irreversible renal failure is most likely to occur in myeloma. In this special situation, where fixed impairment of renal function frequently occurs in association with hypercalcemia, a useful combination is calcitonin and glucocorticoids.[75] These drugs can be used in patients with marked impairment of glomerular filtration rate at the same time as rehydration, and are effective in about 90% of patients with myeloma irrespective of the renal status within 24 hours.

The use of loop diuretics should be restricted to those patients who are in danger of fluid overload. Loop diuretics are not very effective in promoting significant renal calcium excretion, and may provoke volume depletion when used in patients whose volume deficit has not been reversed and who are not fully rehydrated.

The major problem with the treatment of hypercalcemia of malignancy then is not treatment in the initial phase, but rather, in our view, how to manage the patient once the acute episode of hypercalcemia is over. Because of the natural history of the hypercalcemia in patients with malignancy, patients will almost inevitably become hypercalcemic again unless they receive chronic medical therapy for inhibition of bone resorption. Pamidronate is not a particularly convenient drug in this situation, as it must be administered by intravenous infusion, but it is currently the only bisphosphonate that is FDA approved for use in the hypercalcemia of malignancy. Despite the problem with poor oral bioavailability of the bisphosphonates, an alternative possibility for the patient after the initial episode is over is the use of an agent such as alendronate, which can be taken by patients while they are ambulant and out of the hospital. Alendronate has a similar spectrum of activity to pamidronate, which is a closely related drug. Administration of oral amino bisphosphonates, such as pamidronate and alendronate, has been associated with mucosal ulcerations in the mouth and esophagus, and it is important that if alendronate is taken orally, it should be strictly according to the manufacturer's instructions in the package insert. We advise that patients with hypercalcemia of malignancy should be treated in the acute phase with pamidronate and maintained normocalcemic when they leave the hospital with regular intravenous infusions of pamidronate. The FDA has recently approved pamidronate for use in normocalcemic patients with solid tumors and bone metastasis as well as in those patients with myeloma (see below).

MANAGEMENT OF THE NORMOCALCEMIC PATIENT WITH OSTEOLYTIC BONE LESIONS

Most patients with bone metastasis are normocalcemic. In a majority of breast cancer patients with bone metastases, local osteolysis occurs without hypercalcemia,[76] increases in nephrogenous cyclic AMP[35] or PTHrP.[18] Osteolytic bone lesions are most frequent in patients with carcinoma of the breast, carcinoma of the lung and myeloma, the same malignancies that are associated with hypercalcemia. However, there are also other solid tumors where hypercalcemia is rare but osteolytic bone lesions are relatively frequent. These include patients with carcinoma of the thyroid. These patients suffer considerably because of their bone lesions—intractable pain, pathologic fracture after trivial injury, nerve compression syndrome such as spinal cord compression, and of course, propensity to develop hypercalcemia. Until recently we had little to offer these patients. Now, however, the situation has changed considerably.

For patients with myeloma, the FDA had recently approved pamidronate for use in patients with osteolytic lesions who are not hypercalcemic. This is based on a recent study,[77] which shows that intravenous pamidronate given every 4 weeks for nine cycles in almost 400 patients with myeloma caused a significant reduction in skeletal complications (defined as pathologic fracture, requirement for radiation to bone or surgery, or spinal cord compression), decreased the occurrence of new pathologic fractures, and prevented development of hypercalcemia. In addition, this treatment alleviated bone pain and improved quality of life. There was a suggestion in these patients that there may have also been a beneficial effect on overall survival. Therefore, pamidronate is now being widely used early in the course of myeloma because it is a relatively nontoxic drug and may have a beneficial effect not only on bone complications. There is no definitive evidence as yet for a beneficial effect on tumor burden or survival, which will require careful controlled studies in more extended numbers of patients.

Clinical studies have been ongoing for 20 years in normocalcemic patients with solid tumors and osteolytic bone metastases. All of the available evidence from these studies suggest that drugs that decrease bone resorption such as the potent bisphosphonates have a beneficial effect on skeletal complications, including pain and pathologic fracture, prevention of hypercalcemia, and improved quality of life. In a recent multicenter trial of more than 700 patients with stage IV breast cancer with two or more predominantly lytic lesions, with at least one lesion that was 1 cm or greater in diameter, treatment with pamidronate 90 mg intravenously every 3 to 4 weeks for 12 months in conjunction with chemotherapy or hormonal therapy resulted in a significant reduction in skeletal complications and bone pain compared with the control group.[78] However, there may be an added beneficial effect of the bisphosphonates that is even more important. In experimental studies in which human breast cancer cells are inoculated into the left ventricle of the nude mouse, Sasaki et al[79] have shown that bisphosphonates such as risedronate and ibandronate not only prevent the development of skeletal complications and bone metastases, but they also reduce tumor burden in bone. This likely occurs because the bisphosphonates make bone a less favorable environment for the growth of tumor cells by reducing bone turnover and decreasing the supply of local bone-derived growth factors, which also act as tumor growth factors in the bone microenvironment. It is apparent from clinical studies that the use of bisphosphonates reduces significant skeletal morbidity in advanced breast cancer.[80-81] These data suggest that drugs that inhibit bone resorption may be a useful adjuvant therapy in patients with malignant disease by preventing the growth of tumor cells in the skeleton.

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Cancer and Bone*

THERESA A. GUISE AND GREGORY R. MUNDY

Division of Endocrinology, Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7877

I. Introduction

CANCER is associated with significant morbidity in the skeleton. This was evident as early as 1889 when Stephen Paget (1) observed that "in a cancer of the breast the bones suffer in a special way, which cannot be explained by any theory of embolism alone . . . the same thing is seen much more clearly in those cases of cancer of the thyroid body where secondary deposition occurs in the bones with astonishing frequency." He also noted that "a general degradation of the bones sometimes occurs in carcinoma of the breast, yet without any distinct deposition of cancer in them." These early observations were profound as it is now clear that cancer can involve bone through both metastatic and humoral mechanisms.

Since the time of Paget, it has become clear that cancer affects bone in several ways: 1) indirectly through elaboration of factors that act systemically on target organs of bone and kidney to disrupt normal calcium homeostasis; 2) locally and directly via secondary spread of tumor to bone; and 3) via direct involvement by primary bone tumors. As primary bone tumors comprise a small minority of all tumors affecting bone, this review will focus on the aspects of cancer and bone related to the former only.

The three most common neoplasms in humans, breast, prostate, and lung cancer, frequently affect the skeleton. Since the majority of patients dying of cancer have bone involvement either through metastatic spread or as a result of the systemic effects of tumor-produced factors on bone and kidney, this is not a trivial problem. In 1996 alone, the estimated number of new cancer cases in men included 317,000 cases of prostate cancer and 98,900 cases of lung cancer while 184,300 cases of breast cancer and 78,100 cases of lung cancer were diagnosed in women (2). Furthermore, in the same year, prostate and lung cancer were responsible for 41,400 and 94,400 deaths, respectively, in men while breast and lung cancer deaths in women totaled 44,300 and 64,300 individuals, respectively (2). Despite advances in cancer therapy, cancer statistics indicate that the mortality rate of lung cancer is still rising for women, even though 1996 is the first year that it has leveled off for men. Additionally, the age-adjusted death rates of prostate cancer continue to rise, and although 1996 was the first year that a slight decrease in mortality due to breast cancer was observed, the age-adjusted death rate for breast cancer remains similar to that of 1930 (2). Thus, to improve therapy and prevention, it is important to understand the pathophysiology of the effects of cancer on bone as it will be a continued source of morbidity for years to come. Although the topic is an expansive one, this review will attempt to detail scientific advances in this area regarding the pathophysiology of the effects of cancer on bone.

II. Normal Calcium and Bone Homeostasis

As tumor affects bone both through systemic mechanisms as well as via local mechanisms of metastatic spread, the topic of normal bone remodeling and calcium homeostasis will be reviewed.

A. Bone remodeling

Bone is unique among target tissues affected by cancer as it is being continually remodeled under the influence of systemic hormones and local bone-derived growth factors. Bone consists of two physically and biologically distinctive structures. The outer cortical bone is hard mineralized matrix in which cellular and metabolic activities are relatively low. Cortical bone makes up 85% of the total bone in the body and is most abundant in the long bones of the appendicular skeleton. The volume of cortical bone is regulated by the formation of periosteal bone, by remodeling within Haversian systems, and by endosteal bone resorption. Cancellous or trabecular bone constitutes the remaining 15% of the skel-

Address reprint requests to: Theresa A. Guise, M.D., Division of Endocrinology, Department of Medicine, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78284-7877. E-mail: guise@uthscsa.edu.

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eton and is most abundant in the vertebral bodies. The adult skeleton is in a dynamic state as the coordinated actions of osteoclasts and osteoblasts on trabecular surfaces and in Haversian systems result in continual bone resorption and formation. The normal mineralization of bone matrix is contingent upon adequate amounts of vitamin D, calcium, and phosphate. The mineralized bone matrix contains abundant amounts of growth factors, transforming growth factor-β (TGFβ) and insulin-like growth factor II (IGF-II) comprising the majority (3, 4). Such growth factors are released from the bone matrix as a result of osteoclastic bone resorption (5), a component of the normal remodeling process necessary to maintain the structural integrity of bone. The inner portion of bone consists of multicellular bone marrow in which hematopoietic stem cells, stromal cells, and immune cells reside. The hematopoietic stem cells have the potential to differentiate into the blood-forming elements and bone-resorbing osteoclasts, while the stromal cells support the differentiation of the hematopoietic cells as well as from bone-producing osteoblasts. Cells in the bone marrow, stromal and immune in particular, produce cytokines and growth factors that mediate cell-to-cell interactions in autocrine, paracrine, and/or juxtacrine fashions (6). Thus, tumor secretion of hormones that act systemically on bone may disrupt normal calcium homeostasis and bone remodeling to result in hypercalcemia and bone loss while tumor-produced phosphaturic factors may result in osteomalacia. Likewise, once cancer cells arrest in bone, the high concentrations of growth factors and cytokines in the bone microenvironment provide a fertile soil on which the cells can grow. Furthermore, when the tumor cells stimulate osteoclastic bone resorption, this bone microenvironment is even more enriched with bone-derived growth factors that enhance survival of the cancer and similarly disrupt normal bone remodeling to result in bone destruction.

B. Calcium homeostasis

Blood-ionized calcium concentrations are remarkably stable in normal individuals due to a complex regulatory system involving the actions of three calciotropic hormones on the target organs of bone, gut, and kidney. Calcium exchanged between the extracellular fluid and these target organs normally remains in zero balance (Fig. 1). Normal calcium homeostasis is dependent on the interactions of PTH, 1,25-$(OH)_2D_3$, and calcitonin on these organs to maintain the ionized calcium concentration within a very narrow range. Regulation of normal calcium homeostasis has been extensively reviewed by Chattopadhyay *et al.* (7) as well as by Parfitt (8, 9).

1. PTH. PTH is synthesized by the chief cells of the parathyroid gland, and its secretion is highly dependent on the ionized calcium concentration in the extracellular fluid. The serum PTH concentration decreases as the serum calcium concentration increases (Fig. 2) and represents a simple negative feedback loop (10). Similar to other endocrine hormones, such as those secreted by the anterior pituitary, PTH is secreted in a pulsatile fashion in the normal state as well as in states of primary hyperparathyroidism (12). The calcium-sensing receptor that mediates this negative feedback has been cloned from bovine parathyroid cells (13) and is mutated in the disorders of familial benign hypocalciuric hypercalcemia (13–19) and autosomal dominant hypocalcemia (20, 21). Active vitamin D metabolites decrease PTH synthesis *in vitro* and *in vivo* (22, 23) as well.

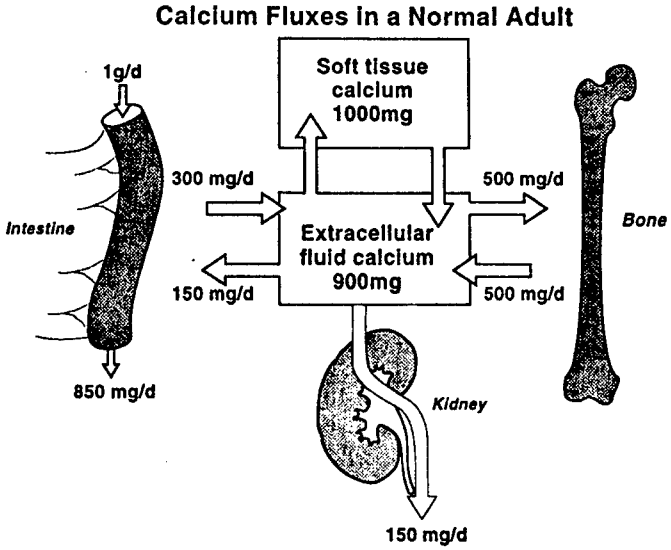

FIG. 1. Calcium homeostasis for a normal adult in zero calcium balance. The numbers are estimates of the amount of calcium exchanged between the extracellular fluid and gut, kidney, and bone each day. The exchange system between bone fluid and the extracellular fluid is not taken into account. [Adapted from G. R. Mundy, Bone Remodeling and Its Disorders (228).]

The biological actions of PTH include: 1) stimulation of osteoclastic bone resorption and release of calcium and phosphate from bone; 2) stimulation of calcium reabsorption and inhibition of phosphate reabsorption from renal tubules; and 3) stimulation of renal production of 1,25-$(OH)_2D_3$, which increases intestinal absorption of calcium and phosphate. The amino terminus of the PTH molecule binds to the PTH receptor, a member of the family of G protein-coupled receptors that contain seven transmembrane-spanning domains (24), to elicit these biological responses. Activating mutations of this receptor have been demonstrated in the rare hypercalcemic disorder of Jansen's metaphyseal dysplasia (25, 26). Recently, other receptors for PTH have been identified. A distinct G protein-coupled receptor that is exclusively activated by PTH, and not PTH-related protein (PTHrP), has been cloned. This PTH-2 receptor is abundant in brain and pancreas, although its function is not yet clear (27). Additionally, functional evidence for a receptor that binds only the carboxyl-terminal portion of PTH exists (28).

Metabolism of PTH is complex, and the intact and biologically active peptide has a half-life of less than 4 min (29). Intact PTH is cleared rapidly by kidney and liver (30–34). Carboxy-terminal fragments circulate significantly longer than the intact hormone, mainly because they are cleared exclusively by glomerular filtration (35, 36). Highly sensitive and specific immunoradiometric assays for intact PTH are now widely available (37) and are extremely useful when employed in the differential diagnosis of hypercalcemia.

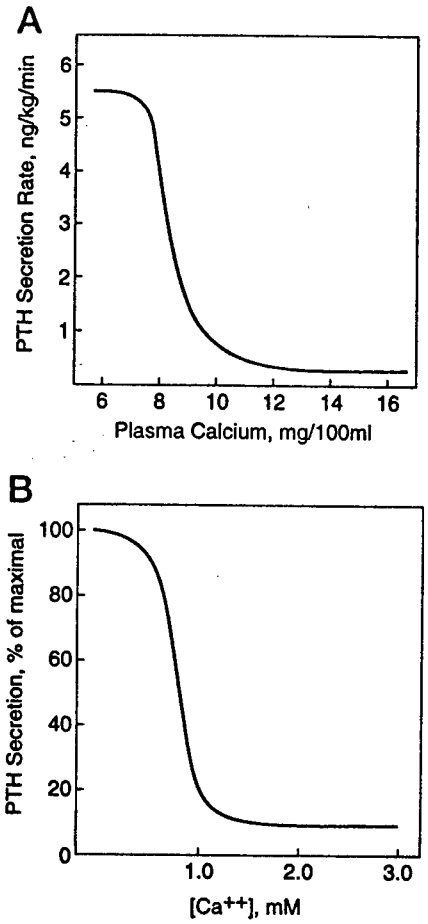

FIG. 2. PTH secretion as a function of calcium *in vivo* and *in vitro*. A, Secretory response of bovine parathyroid glands to induced alterations of plasma calcium. [Modified from G. P. Mayer and J. G. Hurst (11). © The Endocrine Society.] B, PTH secretion by dispersed parathyroid cells in culture as a function of extracellular calcium concentration. [Adapted from E. M. Brown, *Mineral and Electrolyte Metabolism* 8:130–150, 1982, with permission of S. Karger AG, Basel.]

2. *Calcitonin.* Plasma-ionized calcium concentration is the most important regulator of calcitonin secretion (38). Increases in plasma-ionized calcium result in an increase in calcitonin secretion and, conversely, a fall in the ambient calcium concentration inhibits calcitonin secretion. These changes are likely mediated through the calcium-sensing receptor, as the parafollicular cells of the thyroid gland express the same calcium-sensing receptor that is expressed in the parathyroid and kidney (39). Gastrointestinal peptide hormones, gastrin in particular, are potent calcitonin secretagogues. Although the physiological significance of this observation remains unclear, it is the basis for the pentagastrin stimulation test, a provocative test to determine the capacity of a patient to secrete calcitonin (40).

The precise biological role of calcitonin in the overall schema of calcium homeostasis is uncertain. Calcitonin directly inhibits osteoclastic bone resorption (41), and the effect is rapid, occurring within minutes of administration. This inhibition is accompanied by the production of cAMP (42), as well as an increase in cytosolic calcium (43) in the osteoclast, and results in contraction of the osteoclast cell membrane (44). These effects are transient and likely have little role in chronic calcium homeostasis. Clinical observations support this since neither calcitonin-deficient patients (athyroid) nor patients with medullary thyroid cancer and excess calcitonin production experience alterations in calcium homeostasis. The calcitonin receptor (45) is a G protein-coupled receptor with seven-transmembrane domains that is structurally similar to the PTH/PTHrP and secretin receptors. The half-life of calcitonin is measured in minutes and metabolism occurs predominantly in the kidney (38). Clinical abnormalities of calcitonin secretion include medullary thyroid carcinoma, small cell lung cancer, and carcinoids and islet cell tumors of the pancreas.

3. *Calcitriol.* The steroid hormone calcitriol or 1,25-$(OH)_2D_3$ is the major biologically active metabolite of the vitamin D sterol family. Vitamin D precursor (previtamin D_3) is either ingested in the diet or synthesized in the skin from 7-dehydrocholesterol through exposure to sunlight (46, 47). Hydroxylation occurs in the liver at the C-25 position to form 25-hydroxyvitamin D [25(OH)D], the precursor of the more potent metabolite, 1,25-$(OH)_2D_3$. 25(OH)D is hydroxylated at the C-1 position in the kidney by 1α-hydroxylase, a complex cytochrome P_{450} mitochondrial enzyme system located in the proximal nephron (48), to form 1,25-$(OH)_2D_3$ (49–51). The renal 1α-hydroxylation of 25(OH)D is the major recognized control point in vitamin D metabolism, responding to ambient phosphorus, PTH, and calcium concentrations. PTH and low serum phosphate concentrations independently increase 1,25-$(OH)_2D_3$ production, while hypercalcemia and 1,25-$(OH)_2D_3$ inhibit renal 1α-hydroxylase activity. Under physiological conditions, the kidney is the sole source of 1,25-$(OH)_2D_3$. The other known important extrarenal sites of 1,25-$(OH)_2D_3$ production are the placenta and granulomatous tissue (52–54). The half-life of 1,25-$(OH)_2D_3$ in the circulation is approximately 5 h in humans. Fifteen percent is excreted as urinary metabolites and 50% as fecal metabolites.

1,25-$(OH)_2D_3$ increases plasma calcium and phosphate concentrations by increasing the absorption of calcium and phosphate from the gastrointestinal tract (51). It also increases bone resorption (55) and enhances the capacity for PTH to promote renal tubular calcium reabsorption in the nephron. It is a powerful differentiation agent for committed osteoclast precursors (56, 57), causing their maturation to multinucleated cells that are capable of resorbing bone. Thus, 1,25-$(OH)_2D_3$ ensures a supply of calcium and phosphate available at bone surfaces for the mineralization of bone matrix. Deficiency of 1,25-$(OH)_2D_3$ or of 1α-hydroxylase results in osteomalacia or rickets, as does resistance to 1,25-$(OH)_2D_3$, caused by mutations in the vitamin D receptor (58–61). Although the function of other vitamin D metabolites has been unclear, recent evidence from mice deficient in the 24-hydroxylase gene indicate that such metabolites have a role in normal bone metabolism. Deficiency of 24-hydroxylase results in lack of the vitamin D metabolites hydroxylated at the 24 position and abnormal bone structure consisting of accumulation of osteoid at sites of intramembranous ossification (62).

C. Defenses against hyper- and hypocalcemia

The normal physiological defenses against hypercalcemia and hypocalcemia are listed in Table 1. The majority of these defense mechanisms are mediated through the hormonal actions of PTH and 1,25-$(OH)_2D_3$. Although the role of endogenous calcitonin is relatively modest in comparison to PTH and 1,25-$(OH)_2D_3$, pharmacological calcitonin therapy can be beneficial as discussed later.

PTH secretion increases in response to a fall in ionized calcium concentration. This results in 1) osteoclastic bone resorption and release of calcium and phosphate from bone into the extracellular fluid compartment; 2) renal tubular reabsorption of calcium and inhibition of phosphate uptake; and 3) synthesis of 1,25-$(OH)_2D_3$. If these mechanisms are intact, the extracellular calcium concentrations should return to normal.

In the converse situation, a rise in ionized calcium concentration results in decreased PTH secretion from the parathyroid glands. Thus, renal tubular calcium reabsorption is decreased, as is osteoclastic bone resorption. Synthesis of 1,25-$(OH)_2D_3$ and, subsequently, gastrointestinal absorption of dietary calcium and phosphate are decreased. Thus, the normal response to increases in ionized calcium is an increase in renal calcium excretion and a decrease in intestinal absorption of calcium.

In general, these hormonal responses are more effective in protecting against hypocalcemia than hypercalcemia. Perturbations in these mechanisms, as exemplified by excessive increases in bone resorption, deficiencies or excess of PTH or 1,25-$(OH)_2D_3$, and defects in renal capacity to handle calcium and phosphate, will result in either hypercalcemia or hypocalcemia.

III. Humoral Mechanisms by Which Solid Tumors Affect the Skeleton

A. Hypercalcemia

Hypercalcemia is defined as a total serum calcium, adjusted for protein concentration, above 10.2 mg/dl (2.55 mmol/liter) in adults (63). Ionized calcium is a more precise measure of calcium concentration, the normal plasma concentrations ranging from 1.12–1.23 mmol/liter (63). With the advent of automated biochemical testing, hypercalcemia is now recognized to be more common than once realized. By far, the most common causes of hypercalcemia are primary hyperparathyroidism and malignancy.

1. Clinical features of hypercalcemia. The clinical features of hypercalcemia are listed in Table 2. Symptoms may vary in individual patients and are related both to the absolute concentration of serum calcium and to the rate of rise in serum calcium. Symptoms also reflect the underlying cause of the hypercalcemia as well as intercurrent medical conditions. In older or critically ill patients, symptoms of hypercalcemia may be more prominent with relatively small increases in serum calcium concentration. Hypercalcemia most often results in neuromuscular, gastrointestinal, and renal manifestations. Severe hypercalcemia is likely the result of a vicious cycle. The hypercalcemic effects of anorexia, nausea, vomiting, and impaired renal concentrating ability lead to dehydration and, subsequently, altered mental status. This, in turn, may promote immobilization and lead to worsening hypercalcemia. In addition to the symptoms of hypercalcemia, clinical features of hypercalcemia of malignancy include signs and symptoms of the underlying cancer. Generally, the cancer is well advanced when hypercalcemia occurs, and the prognosis is poor. Survival beyond 6 months is uncommon (64–66).

2. Humoral mediators of hypercalcemia in malignancy. Malignancy is the most common cause of hypercalcemia in the hospitalized patient, and malignancy-associated hypercalcemia is one of the more common paraneoplastic syndromes. The relative frequencies of malignancies associated with hypercalcemia are listed in Table 3 (67). Hypercalcemia occurring in the setting of malignancy may be due to 1) humoral factors secreted by tumors that act systemically on target organs of bone, kidney, and intestine to disrupt normal calcium homeostasis; 2) local factors secreted by tumors in bone, either metastatic or hematological, which directly stimulate osteoclastic bone resorption; and 3) coexisting primary hyperparathyroidism.

It is probably more accurate to think of the first two situations as a continual spectrum rather than as discrete groups. The pathophysiology of hypercalcemia is very different in patients with solid tumors and no bone metastases at one end of the spectrum, and myeloma associated with extensive local bone destruction adjacent to the tumor cells at the other. However, in between these two extremes are hypercalcemic patients with squamous cell carcinomas in which hypercalcemia may occur with some, but not extensive, osteolytic bone metastases and hypercalcemic patients with advanced breast carcinoma in which hypercalcemia almost never occurs in the absence of extensive osteolytic bone destruction. Separating hypercalcemia into subcatego-

TABLE 1. Defenses against hypocalcemia and hypercalcemia

Organ	Hypocalcemia	Hypercalcemia
Parathyroid	↑ PTH secretion	↓ PTH secretion
Kidney	↓ GFR → ↓ filtered Ca^{++} ↑ Ca^{++} reabsorption ↑ $1,25(OH)_2D_3$	↑ GFR → ↑ filtered Ca^{++} ↓ Ca^{++} reabsorption ↓ $1,25(OH)_2D_3$
Gastrointestinal tract	↑ Ca^{++} absorption	↓ Ca^{++} absorption
Skeleton	↑ Bone resorption	↓ Bone resorption

TABLE 2. Clinical features of hypercalcemia

Neurologic and psychiatric	Lethargy, drowsiness Confusion, disorientation Disturbed sleep, nightmares Irritability, depression Hypotonia, decreased deep tendon reflexes Stupor, coma
Gastrointestinal	Anorexia, vomiting Constipation Peptic ulceration Acute pancreatitis
Cardiovascular	Arrhythmias Synergism with digoxin ? Hypertension
Renal	Polyuria, polydipsia Hypercalciuria Nephrocalcinosis Impaired glomerular filtration

TABLE 3. Malignancies associated with hypercalcemia (67)

Malignancy	Frequency (%)
Lung	35
Breast	25
Hematologic	14
Head and neck	6
Renal	3
Prostate	3
Unknown primary	7
Others	7

ries based on the assumption that the underlying mechanisms are distinct is not entirely satisfactory because the mediators may be identical except that in one situation it is a local mediator while in another it is a humoral mediator. Additionally, if the tumor burden in bone is great, local tumor-produced mediators of bone resorption may be produced in sufficient quantities to have systemic effects. Although this review will discuss the effects of cancer on bone organized into such subcategories, it is important that the reader understand that a significant portion of cancer patients will fall into the middle of this spectrum, having both humoral and local effects of tumor on bone.

a. PTHrP: Although cancer has been associated with hypercalcemia since the 1920s, it wasn't until 1941 that Fuller Albright first proposed the syndrome of ectopic hormone production in a patient with hypercalcemia, renal carcinoma, and a solitary bone metastasis (68). As the patient had normal parathyroid glands at the time of the neck operation, Albright postulated that the tumor produced PTH but when he had Collip assay the tumor for PTH, it was not detected. Thereafter, the syndrome was often referred to as "pseudohyperparathyroidism" or "ectopic hyperparathyroidism." When more sensitive PTH assays became available, it was clear that the offending factor was not PTH, but rather an immunologically distinct factor that had PTH-like biological activity (69).

Biochemical features of malignancy-associated hypercalcemia share some similarities to those seen in primary hyperparathyroidism (1°HPT). Specifically, in addition to the hypercalcemia, hypophosphatemia can occur (depending on the patient's renal function) as well as hypercalciuria, hyperphosphaturia, and increased excretion of nephrogenous cAMP (69). Plasma-intact PTH concentrations are suppressed in this syndrome except in rare cases of ectopic PTH production or concomitant primary hyperparathyroidism. Plasma 1,25-$(OH)_2D_3$ concentrations are generally low except in certain hematological malignancies when the humoral mediator is 1,25-$(OH)_2D_3$.

Almost 50 yr after Albright's observations, this PTHrP was purified from human lung cancer (70), breast cancer (71), and renal cell carcinoma (72) simultaneously by several independent groups and was cloned shortly thereafter (73). It is now evident that PTHrP, and not PTH, is a major mediator of humoral hypercalcemia of malignancy (74, 75), although four cases of authentic tumor-produced PTH have been reported (76–79). PTHrP has 70% homology to the first 13 amino acids of the N-terminal portion of PTH (73), binds to PTH receptors (80), and shares similar biological activity to PTH (81). Specifically, it stimulates adenylate cyclase in renal and bone systems (71, 72, 81–83), increases renal tubular reabsorption of calcium and osteoclastic bone resorption (82, 83), decreases renal phosphate uptake (81, 82, 84), and stimulates 1α-hydroxylase (81). PTHrP has been found in a variety of tumor types associated with hypercalcemia including squamous, breast, and renal carcinoma (85, 86). Although the majority of squamous cell carcinomas produce PTHrP (87), the capacity to cause hypercalcemia may depend on the level of PTHrP gene expression, which in turn may be determined by differential transcription of the PTHrP gene promoters (88). The regulation of PTHrP is complex, and factors such as PRL (89), epidermal growth factor (EGF) (90–93), insulin (91), IGF-I (91, 94) and II (91), TGFα (95), TGFβ (93, 96–98), angiotensin II (99), stretch (100), and the *src* protooncogene (101) have been shown to increase expression, while glucocorticoids (91, 94, 102, 103) and 1,25-$(OH)_2D_3$ (90, 91) decrease it. Estrogen has been shown to increase PTHrP expression in uterine tissue, and *in vitro* studies suggest that an estrogen response element is present in the PTHrP gene (104, 105). Mutations in codons 248 and 273 of the p53 tumor suppressor gene repress PTHrP gene expression in some squamous cell carcinomas (106). The cell death inhibitor, Bcl-2, is downstream in a signaling pathway that is required for normal skeletal development (107).

The human PTHrP gene is much larger and more complex than the human PTH gene. It spans approximately 15 kb of genomic DNA and has nine exons and three promoters. Three PTHrP isoforms of 139, 141, or 173 amino acids as well as multiple PTHrP mRNA species exist (93). There is considerable sequence homology across species up to amino acid 111 (108). Cell line-specific utilization of the promoters and of the 3'-alternative splicing pathways among bone, breast, kidney, and lung cell lines have been demonstrated (93). In this study, dexamethasone decreased while EGF and TGFβ increased abundance of each of the alternative mRNA species. Furthermore, EGF treatment increased transcription from promoters 1 and 2 and stabilized exon VII- and IX-containing transcripts in various cell lines (93).

Like PTH and other endocrine peptides, PTHrP undergoes

endoproteolytic posttranslational processing that results in several secretory forms: 1) an amino-terminal PTHrP-(1–36); 2) a midregion species that begins at amino acid 38 that has an undefined carboxyl terminus (109, 110); and 3) a carboxyl-terminal species that is recognized by an antibody directed against the 109–138 region (110–112). The preponderance and arrangement of basic residues in the protein sequence suggest that members of the subtilisin family of endoproteases, such as furin (113), PC 1/3, PC-2, PACE-4, and PC8 (114), are responsible for such processing (109, 115, 116). Posttranslational modification of PTHrP also occurs as glycosylation of an amino-terminal PTHrP species produced by keratinocytes has been reported (117). The subject of posttranslational processing of PTHrP, as well as the receptor and signal transduction pathways employed by the mature secretory forms of PTHrP, has been extensively reviewed by Orloff *et al.* (115). Regulation of PTHrP secretion may be cell specific as PTHrP expressed in neuroendocrine cells is secreted in a regulated fashion as compared with a constitutive secretion when expressed in nonneuroendocrine cell types such as squamous cell carcinoma (116). Although PTHrP mediates its calcemic effects through the classic PTH/PTHrP receptor, there is evidence for a separate PTHrP receptor (118). However, the function of such a receptor remains unclear.

PTHrP has been detected in a variety of tumor types as well as in normal tissue (85, 86). The widespread expression of PTHrP in normal tissue was the first evidence that the hormone had a role in normal physiology. In addition to the PTH-like effects, emerging work testifies to the fact that PTHrP has an important role in normal physiology. Such a topic is beyond the scope of this review, but suffice it to say that PTHrP appears to be important in 1) the regulation of cartilage differentiation and bone formation through endochondral ossification (119–121); 2) growth and differentiation of skin (122), mammary gland (123, 124), and pancreatic islets (125); 3) cardiovascular function (126); 4) transepithelial calcium transport in the distal nephron, mammary epithelia, and the placenta (124, 127); 5) relaxation of smooth muscle in uterus, bladder, arteries, stomach, and ileum (99, 128–130); and 6) host immune function (131–133). Bone perichondrial cell production of PTHrP regulates cartilage cell differentiation and has been linked to expression of Indian Hedgehog gene (134). Indian Hedgehog protein expressed by prehypertrophic cartilage cells inhibited cartilage differentiation, and this inhibitory effect was mediated by PTHrP. The normal physiological functions of PTHrP have been extensively reviewed elsewhere (135, 136).

The role of PTHrP in normal breast physiology (137, 138) sheds light on its potential importance in the pathophysiology of hypercalcemia and bone metastasis associated with breast cancer, which is discussed in later portions of this review. PTHrP is expressed in lactating mammary tissue (139) and secreted into milk at concentrations 10,000–100,000 times greater than plasma concentrations of humans with malignancy-associated hypercalcemia (140–144). Increased plasma PTHrP concentrations have been documented in at least two patients with the rare syndrome of lactational hypercalcemia (145–147) in addition to some breast-feeding mothers (148). Thus, PTHrP may be responsible for mobilizing calcium from maternal bone for use in milk production, and it may mediate lactation-associated bone loss.

In addition to the diverse and accumulating normal physiological functions of PTHrP, it likely has a multifunctional role in cancer as well. Such identified functions include 1) mediating hypercalcemia; 2) aiding in the development and progression of osteolytic bone metastasis in breast cancer; 3) regulating growth of cancer cells (149–151); and 4) acting as a cell survival factor (107).

The hypercalcemia of malignancy syndrome was the first identified consequence of the PTHrP effects in cancer. In this syndrome, tumor-produced PTHrP interacts with PTH receptors in bone and kidney to cause hypercalcemia, osteoclast-mediated bone resorption, and increased nephrogenous cAMP and phosphate excretion. The PTH-like properties of PTHrP, and specifically increasing osteoclastic bone resorption and renal tubular calcium reabsorption, appear to be responsible for the hypercalcemia. Approximately 80% of hypercalcemic patients with solid tumors have detectable or increased plasma PTHrP concentrations (111). Plasma PTHrP concentrations, as measured by a sensitive two-site immunoradiometric assay, are low or undetectable in the plasma of normals (152), but as is the situation with PTH, the C-terminal fragment is increased in patients with chronic renal failure (111). In fact, the plasma C-terminal PTHrP concentration increases as the glomerular filtration rate decreases (112).

i. Humoral hypercalcemia of malignancy (HHM) vs. 1°HPT. Despite similarities between HHM and 1°HPT and the similar biological actions of PTHrP and PTH, respectively, unexplained differences between these syndromes exist. First, patients with PTHrP-mediated HHM have low serum concentrations of 1,25-$(OH)_2D_3$ compared with patients with 1°HPT (153), even though both proteins stimulate renal 1α-hydroxylase activity. Clinical studies in which normal humans received short-term infusions of PTHrP-(1–34) (154) or PTHrP-(1–36) (155) revealed increased serum 1,25-dihydroxyvitamin D concentrations comparable to those who had received a similar infusion of PTH-(1–34). Animal studies have revealed similar findings (156, 157). Female nude mice infused with synthetic PTHrP-(1–40) for 7 days developed hypercalcemia, hypophosphatemia, and increased serum 1,25-dihydroxyvitamin D concentrations (157). Likewise, male nude mice bearing Chinese hamster ovarian (CHO) cell tumors transfected with the cDNA for human prepro-PTHrP or prepro-PTH developed similar hypercalcemia and increased plasma concentrations of 1,25-dihydroxyvitamin D when compared with control animals bearing untransfected CHO tumors (158). Additionally, similar increases in blood ionized calcium and 1,25-dihydroxyvitamin D concentrations were observed in nude mice bearing CHO tumors that were engineered to secrete PTHrP mutants truncated at the carboxyl terminus (156).

Second, human studies using either quantitative bone histomorphometry (159) or biochemical markers of bone turnover (160) have demonstrated that although patients with either HHM or 1°HPT have increased osteoclastic bone resorption, many patients with HHM do not have the coupled increase in osteoblastic bone formation that those with 1°HPT have. Serum osteocalcin concentrations, a marker for

bone formation, were significantly increased in patients with 1°HPT compared with normals (161). In the same study, serum osteocalcin concentrations in hypercalcemic patients with bone metastasis were significantly lower compared with those of normal controls, while normocalcemic patients with bone metastases had values similar to normal humans. These osteocalcin concentrations correlated with histomorphometric parameters of bone formation but not bone resorption (161). In the studies by Fraher *et al.* (154) and Everhart-Caye *et al.* (155), in which normal humans received infusions of PTH or PTHrP, bone histomorphometry or biochemical markers of bone turnover were not measured. Such studies done with PTHrP infusions in rodents have revealed increased osteoclastic bone resorption, as well as increased bone formation, as assessed by dynamic bone histomorphometry (157). In contrast, nude mice bearing a PTHrP-secreting human squamous cell carcinoma demonstrated increased bone resorption and decreased bone formation as assessed by dynamic bone histomorphometry (162). Thus, whether PTHrP alone is responsible for the uncoupling of bone formation from bone resorption is an issue that remains controversial.

Unlike the metabolic acidosis seen in patients with primary hyperparathyroidism, patients with malignancy-associated hypercalcemia often have a metabolic alkalosis with a low plasma chloride and high plasma bicarbonate concentration. Although many explanations have been postulated for the discrepancies between HHM and 1°HPT, such as differences between the pulsatile secretion of PTH and the presumed continuous secretion of PTHrP, suppression of bone formation and 1α-hydroxylase activity by other tumor-associated factors, biologically active PTHrP fragments, or hypercalcemia *per se* (163), the reasons for these differences have not been adequately elucidated.

ii. Modulation of PTHrP effects by other tumor-associated factors. Regardless of the reasons for the clinical differences between HHM and 1°HPT, there is clear evidence that other tumor-produced factors can modulate the end-organ effects of PTHrP as well as its secretion from tumors. Using an *in vivo* model of PTH and PTHrP-mediated hypercalcemia, Uy *et al.* (164) demonstrated that both proteins, when produced by tumors in which the corresponding genes were transfected and then inoculated into nude mice, caused similar hypercalcemia as well as increases in osteoclastic bone resorption, more committed marrow mononuclear osteoclast precursors, and mature osteoclasts. No stimulatory effects were seen on the multipotent osteoclast precursors, the granulocyte/macrophage colony-forming unit. In a similar model system, IL-6 potentiated the hypercalcemia and bone resorption mediated by PTHrP *in vivo* by stimulating production of early osteoclast precursors (165). Likewise, TGFα has been shown to enhance the hypercalcemic effects of PTHrP in an animal model of malignancy-associated hypercalcemia (166) as well as to modulate the renal and bone effects of PTHrP (167, 168). Sato *et al.* (169) demonstrated that IL-1α and PTHrP may have synergistic effects *in vivo*, and others have shown that IL-1 may modulate the renal effects of PTHrP (170). Finally, Uy *et al.* have demonstrated that tumor necrosis factor-α (TNFα) enhanced the hypercalcemic effect of PTHrP by increasing the pool of committed osteoclast progenitors with a subsequent increase in osteoclastic bone resorption. Bone formation parameters in these nude mice indicate that TNFα did not inhibit the new bone formation stimulated by PTHrP (171).

Such tumor-associated factors also appear to be important regulators of PTHrP expression and secretion by tumors. EGF has been shown to stimulate PTHrP expression in a keratinocyte cell line (172) as well as a mammary epithelial line (91) while TGFα enhanced PTHrP expression in a human squamous cell carcinoma of the lung (173). Interleukin-6 (IL-6), TNF, IGF-I, and IGF-II increased the production of PTHrP *in vitro* by a human squamous cell carcinoma (94). TGFβ, which is abundant in bone, released in active form by resorbing bone and expressed by some breast cancers (174, 175) and cancer-associated stromal cells (176), has been shown to enhance secretion of and stabilize the message for PTHrP in a renal cell carcinoma (96) as well as in a squamous cell carcinoma (97, 98). Other data (177, 178) demonstrate that this relationship also exists in a human breast adenocarcinoma cell line, MDA-MB-231.

iii. PTHrP in hypercalcemia associated with breast cancer. Hypercalcemia in breast cancer represents a special situation. Although it is clear that the predominant way in which breast cancer affects bone is through metastatic mechanisms, there is sufficient evidence to support the notion that breast cancers may secrete factors that act systemically to stimulate osteoclastic bone resorption and to increase renal tubular reabsorption of calcium (179–181). Hypercalcemia is associated with breast cancer, occurring in approximately 10% of afflicted women during the course of their disease (182). It is likely more common in those with advanced breast cancer. Osteoclast-mediated skeletal destruction by metastatic tumor is a major mechanism responsible for hypercalcemia, as increased osteoclastic bone resorption has been documented histologically in areas surrounding breast cancer metastases (183–186). However, humoral mechanisms may contribute in 10–60% of cases of breast cancer-associated hypercalcemia as evidenced by increased nephrogenous cAMP and plasma PTHrP in some patients (186–190).

PTHrP is clearly a significant factor in mediating hypercalcemia in breast cancer (191). Since PTHrP is expressed in normal breast tissue and appears to play an important role in normal breast physiology, its overproduction in breast cancer is not surprising. One of the three tumors from which PTHrP was originally purified was a breast cancer from a patient with humoral hypercalcemia of malignancy (70). PTHrP was detected by immunohistochemical staining in 60% of 102 invasive breast tumors removed from normocalcemic women, but not in normal breast tissue (192). At least four other studies have confirmed these percentages (193–196), and one of these has demonstrated immunoreactive PTHrP within the cytoplasm of lobular and ductal epithelial cells in normal and fibrocystic breast tissues (193). Furthermore, 65–92% of hypercalcemic breast cancer patients (with and without bone metastasis) had detectable plasma PTHrP concentrations by RIA similar to those documented in patients with humoral hypercalcemia of malignancy due to nonbreast tumors (180, 195). Not only is PTHrP an important mediator of hypercalcemia in breast cancer, it may have a significant role in the pathophysiology of breast cancer me-

tastasis to bone as evidenced by the clinical studies indicating that PTHrP expression by the primary breast cancer is more commonly associated with the development of bone metastasis and hypercalcemia (195). This topic will be discussed in a later section.

iv. PTHrP in hypercalcemia associated with hematological malignancies. The mechanisms responsible for hypercalcemia associated with hematological malignancies are multifactorial and include secretion of local bone-active cytokines, such as IL-6, IL-1, and lymphotoxin or TNFβ, from tumor in bone or from systemic effects of tumor-produced factors such as 1,25-$(OH)_2D_3$ (discussed below). Recent data from a clinical study of 76 patients with various hematological malignancies demonstrate that PTHrP also may be an important pathogenetic factor in the development of hypercalcemia in some patients (197). In this study, eight of the 14 hypercalcemic patients had non-Hodgkin's lymphoma and, of these, 62% had significant increases in plasma PTHrP concentrations. The serum 1,25-$(OH)_2D_3$ concentrations, when measured, were low in the hypercalcemic non-Hodgkin's lymphoma patients who had increased plasma PTHrP concentrations (197). Additionally, one of two hypercalcemic patients with Hodgkin's disease and one of four hypercalcemic patients with multiple myeloma had increased plasma PTHrP concentrations. Also of interest in this study is the fact that several normocalcemic patients with non-Hodgkin's lymphoma, Hodgkin's lymphoma, multiple myeloma, and Waldenstrom's macroglobulinemia had increased plasma PTHrP concentrations as measured by an amino-terminal PTHrP assay (197). Using a sensitive two-site immunoradiometric assay, other investigators have noted increased plasma PTHrP concentrations in patients with adult T cell leukemia and B cell lymphoma (198). Finally, circulating concentrations of PTHrP, comparable to those in humoral hypercalcemia of malignancy, were present in two of four hypercalcemic patients with non-Hodgkin's lymphoma, in three of nine with myeloma (199), and in a patient with myeloid blast crisis of chronic myeloid leukemia (200). Thus, the humoral mediators in the hypercalcemia associated with hematological malignancies include both 1,25-$(OH)_2D_3$ and PTHrP.

b. 1,25-$(OH)_2D_3$: In the setting of hypercalcemia, serum concentrations of 1,25-$(OH)_2D_3$ are normally suppressed unless an autonomous source of PTH is the cause, such as a parathyroid adenoma. Lack of 1,25-$(OH)_2D_3$ suppression in this situation is evidence of disordered regulation of 1,25-$(OH)_2D_3$ synthesis and indicates extrarenal production such as that observed in the hypercalcemia associated with granulomatous disease. Less commonly, tumors may secrete other humoral factors responsible for hypercalcemia. A major mediator of hypercalcemia in Hodgkin's disease, non-Hodgkin's lymphoma, and other hematological malignancies appears to be extrarenal production of 1,25-$(OH)_2D_3$ (201). The mechanism is similar to that observed in hypercalcemia associated with granulomatous disease in which activated macrophages within the granuloma synthesize 1,25-$(OH)_2D_3$ (52, 202, 203). In this scenario, patients usually have increased plasma 1,25-$(OH)_2D_3$ concentrations in addition to low or normal plasma PTH and urinary cAMP concentrations (204) in the absence of bone involvement. In similar studies, affected patients have also been shown to have increased fasting urinary calcium excretion (204) as well as increased intestinal calcium (^{47}Ca) absorption (205). Increased 1,25-$(OH)_2D_3$ concentrations were noted in 12 of 22 hypercalcemic patients with non-Hodgkin's lymphoma. In addition, 71% of 22 normocalcemic patients with non-Hodgkin's lymphoma were hypercalciuric, and 18% had increased serum 1,25-$(OH)_2D_3$ concentrations. These findings led the investigators to conclude that dysregulated 1,25-$(OH)_2D_3$ production is common in patients with diffuse large cell lymphoma (201).

Thus, the mechanisms responsible for hypercalcemia in this setting appear to be multifactorial and include increased intestinal calcium absorption as well as increased osteoclastic bone resorption. Additionally, many of the reported patients had altered renal function, a finding that suggests that impaired renal calcium clearance may also be contributing to the hypercalcemia in certain patients. The low serum PTH and urinary cAMP concentrations indicate that neither PTH nor PTHrP mediates the hypercalcemia in this setting. Prostaglandins, when measured, have been low, and selected patients had no calcium-lowering effect from indomethacin therapy (199). It is likely that the lymphoma tissue itself hydroxylates 25-hydroxyvitamin D to the active 1,25-$(OH)_2D_3$ similar to the situation in hypercalcemia associated with granulomatous disease (52, 203). One α-hydroxylase activity has been demonstrated in human T cell lymphotrophic virus type I-transformed lymphocytes (206). None of the reported patients with 1,25-$(OH)_2D_3$-mediated hypercalcemia had concomitant granulomatous disease, and hypercalcemia often improved with medical or surgical therapy that resulted in a decrease in serum 1,25-$(OH)_2D_3$ concentrations. Recurrence of hypercalcemia and increased plasma 1,25-$(OH)_2D_3$ concentrations has been documented with recurrence of disease (207).

c. PTH: After Fuller Albright's observations in 1941, it was postulated that malignancy-associated hypercalcemia was due to tumor production of PTH. This notion was strengthened by the early PTH RIA data in hypercalcemic patients with malignancy, which suggested that tumors produced factors recognized in these PTH RIAs (208–211). Although, for many years, malignancy-associated hypercalcemia was attributed to ectopic tumor-produced PTH, it is now clear that PTHrP is responsible for most cases. Analysis of 13 human and three animal nonparathyroid tumors of diverse origin associated with hypercalcemia did not detect PTH RNA transcripts (212). Since that time, four cases of authentic tumor-produced PTH have been convincingly demonstrated in a small cell carcinoma of the lung (78), an ovarian cancer (77), a widely metastatic primitive neuroectodermal tumor (76), and a thymoma (79). Molecular analysis of the ovarian carcinoma revealed both DNA amplification and rearrangement in the upstream regulatory region of the PTH gene (77). Interestingly, the primitive neuroectodermal tumor produced both PTH and PTHrP that resulted in severe hypercalcemia (76). These reported patients did not have coexisting primary hyperparathyroidism since the parathyroid glands were normal at the time of neck exploration or at autopsy in all cases. However, the fact remains that ectopic production of PTH is a rare event, and it is clearly docu-

mented that most patients with malignancy-associated hypercalcemia have suppressed plasma PTH concentrations (69). It should be emphasized that the most likely cause of hypercalcemia in the setting of malignancy that is associated with a normal or increased serum PTH concentration is coexisting primary hyperparathyroidism.

d. Other tumor-associated factors: There is accumulating evidence that solid tumors may produce other factors, alone or in combination with PTHrP, that have the capacity to stimulate osteoclastic bone resorption and cause hypercalcemia (213). These factors include IL-1, IL-6, TGFα, and tumor necrosis factor (TNF). Administration of IL-1 injections to mice caused mild hypercalcemia (214, 215), and this IL-1-induced hypercalcemia has been effectively blocked by the IL-1 receptor antagonist (216). Mice bearing CHO tumors transfected with the cDNA for IL-6 developed mild hypercalcemia (217) as did mice bearing a renal carcinoma that cosecreted IL-6 and PTHrP (218). Human TGFα and TNFα have been demonstrated to stimulate osteoclastic bone resorption in vitro and cause hypercalcemia *in vivo* (219–224). TNFα also caused hypercalciuria, without an increase in nephrogenous cAMP, and increased osteoclastic bone resorption *in vivo* in a mouse model (225). In addition, as noted in the previous section, some of these factors have been shown to modulate the end-organ effects of PTHrP on bone and kidney. In some instances, factors such as TGFα, IL-1, IL-6, and TNF enhance the hypercalcemic effects of PTHrP. The ability of IL-6 to enhance PTHrP-mediated hypercalcemia appears to be due to increased production of the early osteoclast precursor, granulocyte macrophage colony forming units, by IL-6 in combination with increased production of the more committed osteoclast precursors stimulated by PTHrP (165). Figure 3 summarizes the known effect of various tumor-produced factors on stages of the osteoclast lineage as determined in bone marrow cultures from mice treated with respective factors (164, 165, 171, 226).

Prostaglandins of the E series are powerful stimulators of bone resorption (227) although their role in bone destruction associated with malignancy remains unclear (228). Some of the effects of cytokines on bone may be mediated in part through prostaglandins as indomethacin, a prostaglandin synthesis inhibitor, has been shown to block part of the osteoclast-stimulatory effects of IL-1 *in vivo* (214, 215). Although prostaglandins have been demonstrated to be produced by cultured tumor cells *in vitro*, indomethacin treatment of malignancy-associated hypercalcemia is only occasionally effective (229). Thus, it is unlikely that prostaglandins have a major causal role in hypercalcemia associated with malignancy.

3. Treatment of hypercalcemia associated with malignancy. Treatment of hypercalcemia due to malignancy should always involve treating the underlying tumor. Unfortunately, since this is often not effective or cannot be accomplished with the rapidity needed when the patient is faced with life-threatening hypercalcemia, therapy should also be directed against the mechanisms responsible for the hypercalcemia. In essentially all patients with hypercalcemia of malignancy, there is an increase in osteoclastic bone resorption, and in many there is also an increase in renal tubular calcium reabsorption, even in malignancies that are not associated with PTHrP production (230). Medical therapy is therefore aimed at inhibiting bone resorption and promoting renal calcium excretion. Because hypercalcemia associated with cancer is often accompanied by dehydration, volume expansion with isotonic saline is essential. This serves to increase the glomerular filtration rate and reduces the fractional reabsorption of both sodium and calcium. Since hydration alone will normalize serum calcium concentrations only transiently (231), inhibitors of bone resorption such as the bisphosphonates or calcitonin should be administered as well. When possible, mechanism-specific treatment should be attempted (232). Glucocorticoids, for example, are more effective in reducing the serum calcium concentration in hematological malignancies and 1,25-$(OH)_2D_3$-mediated hyper-calcemia than in solid tumors. Dietary calcium restriction is ineffective in reducing serum calcium concentrations except in cases of vitamin D-mediated hypercalcemia. In these cases, dietary calcium should be restricted to 400 mg daily until the underlying disorder is corrected. It is not desirable or advantageous to reduce calcium intake in hypercalcemia due to malignancy.

Bisphosphonates, analogs of pyrophosphate, have become the most useful antiresorptive agents among the currently available armamentarium for the treatment of hypercalcemia. They have a high affinity for hydroxyapatite in bone and concentrate in areas of high bone turnover. The mechanisms by which bisphosphonates inhibit bone resorption are not clearly understood, but potentially include induction of osteoclast apoptosis, inhibition of osteoclast formation and recruitment, or stimulation of osteoblasts to produce an inhibitor of osteoclast formation (233, 234). Another mechanism by

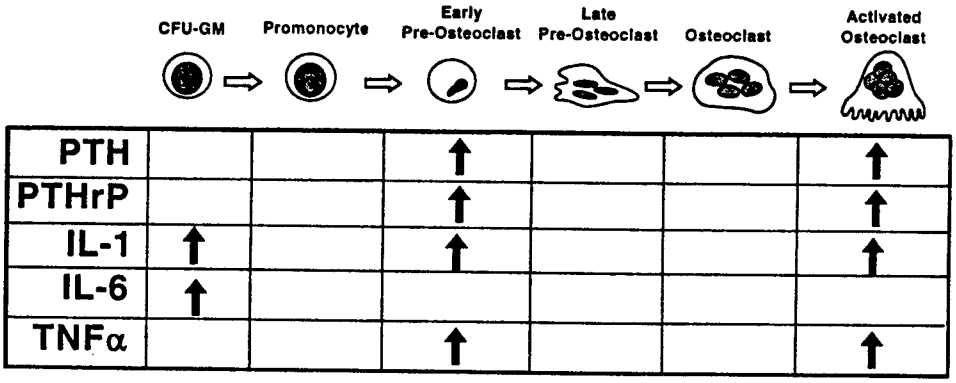

FIG. 3. Effect of tumor-produced factors on cells of the osteoclast lineage *in vivo* (161, 162, 168, 219). Factors were administered to mice via tumor production or injection.

which bisphosphonates might affect bone resorption is by decreasing the function of the osteoclast with respect to attachment and ruffled border formation (233). Recent *in vitro* findings using rodent marrow cultures suggest that tyrosine phosphatase activity is important in osteoclast formation and function and is a potential molecular target of bisphosphonate action (235). Bisphosphonates also inhibit axenic growth of amoebe of the slime mold *Dictyostelium discoideum*, and this property of growth inhibition paralleled the potency of inhibition of bone resorption (236). These findings indicate that bisphophonates may have a mechanism of action that is similar in both the osteoclast and *Dictyostelium discoideum*.

Bisphosphonates vary in potency but, in general, are poorly absorbed and are most effective in treating hypercalcemia when given intravenously. Bisphosphonates are concentrated in bone and remain there until the bone is resorbed. Etidronate, the first available bisphosphonate in the United States, is the least potent. Intravenous etidronate, given in doses of 7.5 mg/kg iv over 3 consecutive days normalized calcium concentration in 30%–40% of patients (237–239). Oral etidronate is generally ineffective in treating hypercalcemia (229), and at sustained dosages of 25 mg/kg per day for more than 6 months, it can cause bone mineralization defects (234). Etidronate can also cause hyperphosphatemia which, in addition to hypercalcemia, may lead to a high calcium-phosphate solubility product.

Pamidronate is a potent aminobisphosphonate available for the treatment of hypercalcemia of malignancy. The drug combines high efficacy with low toxicity profile and thus has become the current bisphosphonate of choice for the treatment of hypercalcemia of malignancy. It is highly effective in normalizing serum calcium concentrations and, when used in dosages recommended for hypercalcemia of malignancy, is not associated with bone mineralization defects. Pamidronate, administered as a single 24-h infusion, normalized serum calcium concentrations in 30% of patients who received 30 mg, 61% of patients who received 60 mg, and 100% of patients who received 90 mg (66). Successful therapy with bisphosphonates is associated with an increase in the plasma PTH and 1,25-$(OH)_2D_3$ concentrations as well as a decrease in the biochemical markers of bone resorption (66, 153, 190). Clinical studies of pamidronate treatment in patients with hypercalcemia of malignancy indicate that the calcium-lowering response to bisphosphonates correlates positively with the presence of bone metastases (240–242) and correlates negatively with plasma PTHrP concentrations (65, 240, 241). Such a relationship has also been demonstrated with clodronate, an oral bisphosphonate (243). This is presumably due to the effects of PTHrP to increase renal tubular reabsorption of calcium, which are not blocked by bisphosphonates. Nonetheless, pamidronate compares favorably to other inhibitors of bone resorption such as plicamycin, calcitonin, and gallium nitrate and is well tolerated. Pamidronate should be delivered as an intravenous infusion over 4–24 h. Clinical studies using 90 mg infusion of pamidronate over 4 h indicate that the mean time to achieve normocalcemia is approximately 4 days while the mean duration of normocalcemia is 28 days (244). Similarly, intravenous pamidronate, 60 mg every 2 weeks, maintained normocalcemia in a majority of patients with malignancy-associated hypercalcemia (245). An effective method for achieving more rapid reduction of the serum calcium is to use the combination of calcitonin and pamidronate (246). Calcitonin acts rapidly to lower the serum calcium, although usually its effects are only transient. Although escape from calcitonin therapy may occur within 48 h, by that time pamidronate is beginning to exert its maximal effects. Calcitonin can be administered either intramuscularly or subcutaneously every 12 h in doses of 200–400 MRC units. Reported side effects of pamidronate include transient low-grade fever and asymptomatic mild hypocalcemia (66). Bone mineralization defects have been reported only in patients receiving high-dose pamidronate at weekly intervals for the treatment of Paget's disease (247). It is possible that other bisphosphonates, such as alendronate, risedronate, and tiludronate, will be effective oral therapy for hypercalcemia of malignancy. Due to its propensity to cause mouth ulcers, oral pamidronate, although effective, is not likely to be approved for such use in the United States.

Calcitonin inhibits osteoclastic bone resorption and renal tubular reabsorption of calcium. The main advantages of calcitonin are its rapid onset of action and its relative lack of serious side effects. Unfortunately, calcitonin alone only transiently normalizes the calcium concentration in patients with hypercalcemia of malignancy. Tachyphylaxis, probably due to down-regulation of calcitonin receptors, frequently develops with calcitonin administration, although this can be delayed with concomitant glucocorticoid treatment (248). However, calcitonin use can be particularly effective in the setting of severe hypercalcemia while waiting for the more sustained hypocalcemic effect of administered bisphosphonates to occur. Calcitonin use with bisphosphonates lowers calcium concentrations more quickly and effectively than either alone (249). Although human calcitonin is available, salmon calcitonin is generally used. If salmon calcitonin is used, a test dose of 1 MRC unit should be administered first, since rare anaphylactic reactions have been reported (250).

Plicamycin, or mithramycin, an antineoplastic agent used in the treatment of certain embryonal cancers (251), is also a potent inhibitor of bone resorption. Plicamycin inhibits DNA-dependent RNA synthesis (252) in tumor cells by binding to the promoter regions on DNA, thus preventing transcription (253). Presumably, osteoclastic bone resorption is inhibited by this mechanism as well. The dosage used to treat hypercalcemia (25 μg/kg) is one-tenth of the usual chemotherapeutic dose and should be infused over 4 h. Although plicamycin is almost invariably effective in lowering serum calcium concentrations, its considerable toxicity has limited its use in more recent years as more potent bisphosphonates have become available. Plicamycin has serious hepato- and nephrotoxicity in addition to local irritation and thrombocytopenic effects, which can limit its use in cancer patients as well as in those with renal impairment.

Gallium nitrate is another antineoplastic agent that, like plicamycin, was found to induce hypocalcemia in normocalcemic cancer patients receiving it (254). It inhibits osteoclastic bone resorption and appears to be more effective in lowering serum calcium concentration than calcitonin (255) and etidronate. Gallium is administered as a continuous infusion over 5 days, making it somewhat less convenient than some other antihypercalcemic agents. Gallium is ex-

creted unchanged by the kidneys and has significant nephrotoxicity. Thus, it should not be administered to patients with renal impairment or to those receiving other nephrotoxic drugs (256).

About 30% of patients treated with glucocorticoids for hypercalcemia associated with nonparathyroid malignancy respond with a fall in calcium concentration (229). However, the response is often not complete and the responsiveness to glucocorticoids is unpredictable (229). Glucocorticoids are most effective in hypercalcemic patients with hematological malignancies, multiple myeloma in particular, as well as in vitamin D-associated disorders such as lymphomas. In hematological malignancies, glucocorticoids inhibit osteoclastic bone resorption by decreasing tumor production of locally active cytokines in addition to having direct tumorolytic effects (257). Glucocorticoids in dosage equivalents of prednisone, 40 to 60 mg daily, should be given for 10 days. If the calcium has not decreased in this period, glucocorticoids should be discontinued. Long-term adverse effects of glucocorticoids, such as osteopenia and Cushing's syndrome, occur with continued administration over several months. This is usually not a consideration in patients with widespread malignancy, who have a very limited prognosis.

B. Oncogenic osteomalacia

Oncogenic osteomalacia is a rare tumor-associated disorder, first recognized in 1947 (258, 259), that is characterized by hypophosphatemia, phosphaturia, normocalcemia, and osteomalacia in the absence of a family history of rickets, heavy metal poisoning, or Fanconi's syndrome. The serum alkaline phosphatase concentration is increased and the serum 1,25-$(OH)_2D_3$ concentration is decreased. Affected patients typically present with bone pain, proximal muscle weakness, and fractures. The disorder may manifest as rickets if it occurs before fusion of the growth plate. Tumors associated with this disorder are generally of mesenchymal origin, small, and benign, although it has occasionally been associated with malignant tumors. Reported tumor types include sclerosing hemangioma (260, 261), paraganglioma (262), prostate cancer (263, 264), oat cell carcinoma of the lung (265), fibrous dysplasia, hemangiopericytoma, osteosarcoma, chondroblastoma, chondromyxoid fibroma, malignant fibrous histiocytoma, giant cell tumor (266), and a metaphyseal fibrous defect (267). Oncogenic osteomalacia associated with metastatic prostate cancer comprises about 10% of all reported cases (264). Regardless of the origin, tumors causing oncogenic osteomalacia are often small and difficult to locate. Some reported locations include the groin, nasopharynx, and the popliteal region. Biochemical abnormalities resolve after complete tumor resection and recur with tumor regrowth. Serum phosphate concentrations increase immediately in the postoperative period while alkaline phosphatase concentrations may take more than 1 yr to normalize with healing of the osteomalacia (260, 262). In one patient, the bone mineral density measurement increased from a preoperative value of 0.627 g/cm^2 to 1.097 g/cm^2 198 days postoperative (262).

Phosphaturia may contribute to the osteomalacia in affected patients as does the apparent decrease in plasma 1,25-$(OH)_2D_3$ concentrations, both of which presumably lower the concentrations of available phosphate ions at the mineralizing bone site. The responsible phosphaturic factor has not been identified, but does not appear to be PTH or PTHrP since calcium and nephrogenous cAMP concentrations are normal in affected patients. Conditioned medium from cell culture of oncogenic osteomalacia tumors has been shown to inhibit phosphate uptake in cultured epithelial opossum kidney cells (260, 262). In one report, there was no measurable immunoactive PTH or PTHrP in the conditioned media from a paraganglioma (262). Another report found that conditioned media from a hemangioma inhibited phosphate uptake in opossum kidney cells without increasing cellular concentrations of cAMP. The media contained PTH-like immunoreactivity without PTHrP immunoreactivity, and the inhibition of phosphate transport was not blocked by a PTH antagonist (260). This putative factor appeared to be heat sensitive and of a molecular mass between 8 and 25 kDa (260).

Serum 1,25-$(OH)_2D_3$ concentrations are low in patients with oncogenic osteomalacia, despite the presence of hypophosphatemia, which normally increases 1,25-$(OH)_2D_3$ production by stimulating renal 1α-hydroxylase activity independent of PTH. Additionally, most reported cases of oncogenic osteomalacia have normal serum 25-hydroxyvitamin D concentrations. Deficient production of 1,25-$(OH)_2D_3$ could be a contributing factor to the pathogenesis of oncogenic osteomalacia in these patients as the clinical and biochemical abnormalities improve during calcitriol therapy in some patients. The pathophysiology of the vitamin D derangement is not well understood, but the clinical features have led investigators to hypothesize that tumor-produced factors inhibit 1α-hydroxylase activity. In one study, tumor extracts from a hemangiopericytoma inhibited the formation of 1,25-$(OH)_2D_3$, and transplantation of this tumor into athymic mice resulted in renal phosphate wasting and decreased 1,25-$(OH)_2D_3$ concentrations (268).

C. Tumor lysis syndrome

Another disorder of calcium homeostasis associated with malignancy is the tumor lysis syndrome, which may occur as a consequence of successful therapy of neoplastic disease (269). The syndrome often occurs during therapy of hematological malignancies, particularly high-grade lymphomas, in which a large number of tumor cells are lysed in a short period of time. It has also been reported during therapy of small cell carcinoma of the lung (270), breast cancer, and medulloblastoma as well as during immunotherapy for sarcoma (271) and therapy with TNFα and monoclonal antibody against GD3 ganglioside in metastatic melanoma (272). Rare reports of spontaneous tumor lysis have also been reported (273). The release of tumoral intracellular ions, such as phosphate and potassium, into the extracellular fluid result in hyperphosphatemia. Hypocalcemia, hyperuricema (due to uric acid release from lysed cells), and renal failure are a consequence.

IV. Local Mechanisms by Which Tumors Affect the Skeleton

It is clear that hypercalcemia associated with cancer conveys a poor prognosis, with survival of less than 3 months (64, 65). However, the majority of patients with metastatic bone disease are not hypercalcemic and, in the case of breast cancer, patients may survive up to 90 months after the detection of the first bone metastases (268, 274). Autopsy studies reveal bone metastasis in 70% of women who died of breast cancer (275–277). Thus, it behooves us to understand the mechanisms responsible for this complication of cancer to effectively decrease the associated morbidity or to prevent it altogether.

A. Clinical manifestations

Both solid tumors and hematological malignancies frequently affect the most vascular areas of the skeleton, specifically in the red bone marrow of the axial skeleton, the proximal ends of the long bones, ribs, and the vertebral bodies. The most common way in which cancer affects the skeleton is directly through local tumor-mediated stimulation of osteoclastic bone resorption. Such osteolytic bone lesions are typical of breast and lung carcinoma as well as hematological malignancies such as multiple myeloma. Although breast cancer cells have been shown to resorb bone directly *in vitro* (278), most evidence [scanning electron microscopic examination of adjacent bone surfaces (184) and response to osteoclast inhibitors] is consistent with the notion that factors secreted by cancer cells can activate osteoclasts locally. This is illustrated in Fig. 4, a photomicrograph of an osteolytic lesion due to the human breast cancer cell line MDA-MB-231. Thus, local and systemic effects of cancer on bone are mediated through one common final pathway, the osteoclast. The resulting osteolytic bone destruction can lead to pain, pathological fractures, nerve compression syndromes, and hypercalcemia.

Tumor in bone may stimulate new bone formation to result in osteoblastic bone metastasis. This is most often associated with prostate cancer, although it less frequently occurs in breast cancer and rarely in a sclerotic variant of myeloma (279) as well as in other malignancies. Osteoblastic metastases are also associated with bone pain and nerve compression syndromes, but unlike osteolytic metastases, this type of bone involvement can cause hypocalcemia (280, 281). Pathological fractures can occur with osteoblastic metastases as well as due to the intrinsically low strength of the new woven bone and/or concomitant osteolysis. In general, sites of pathological fractures commonly include the vertebral bodies and the proximal ends of the long bones. Spinal cord compression is a catastrophic event often associated with metastatic bone disease and can be due to tumor directly impinging on the spinal cord, fracture of vertebral body consumed by destructive osteolytic lesions, or bony overgrowth of osteoblastic lesions.

Hypercalcemia associated with metastatic bone disease or hematological malignancies has been referred to as local osteolytic hypercalcemia, as clinical studies have demonstrated that many patients with bone involvement and hypercalcemia did not have the increase in plasma PTHrP or nephrogeneous cAMP concentration observed in patients with humoral hypercalcemia of malignancy (69, 111). The mechanism of hypercalcemia in this situation was postulated to be local tumor production of factors that stimulate osteoclastic bone resorption, such as TNFβ, IL-6, and IL-1 as in the case of myeloma. However, it is now clear that humoral mediators of hypercalcemia, such as PTHrP, may mediate local osteolysis, even in the absence of hypercalcemia and increased plasma PTHrP concentrations (282). Additionally, if tumor burden in bone is great enough, tumor-produced

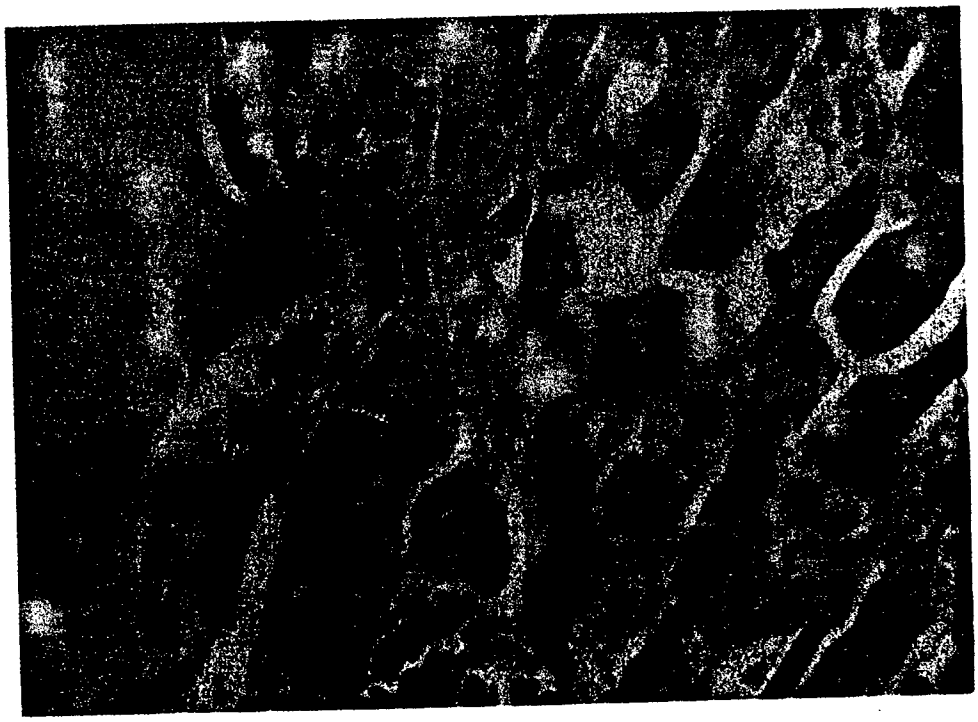

FIG. 4. Photomicrograph of an osteolytic bone lesion. The section was taken from an affected femur of a mouse inoculated into the left cardiac ventricle with the human breast cancer cell line MDA-MB-231. Radiographic appearance of the lesion appears in Fig. 6. Magnification 100×.

factors in bone may be produced in enough quantity locally to reach the systemic circulation and have effects on sites distant from affected bone.

B. Pathophysiology of the metastatic process to bone

Breast and prostate cancer are the most common malignancies in which bone metastases occur. Breast cancer is most often associated with osteolytic metastasis while osteoblastic metastases are more often manifest in prostate cancer. Mixed osteolytic and osteoblastic lesions are often evident in both breast and prostate cancer. The remainder of the review will focus on general principles of metastasis to bone, followed by mechanisms specific to osteolytic and osteoblastic metastasis, citing examples from current research in breast and prostate cancer, respectively. This will be followed by a review of bone involvement in myeloma. The reader should understand that breast and prostate cancer, although predominantly lytic and blastic, respectively, often have components of both osteolysis and osteosclerosis. In this review, delineation of osteolytic and osteoblastic mechanisms of bone metastasis to breast and prostate cancer, respectively, is by no means meant to be exclusive. It is likely that both mechanisms are often operative in the same patient.

1. Anatomical. Tumor metastasis to bone is not a random event, but rather a result of anatomical factors, tumor cell phenotype, and suitability of the metastatic site for tumor growth. Blood flow from the primary site is a significant determinant of the site of metastasis. Studies by Batson (283) describe in detail a low-pressure, high-volume system of valveless vertebral veins that communicate between the spine and intercostal veins independently of the pulmonary, caval, or portal systems. Batson accessed this plexus in cadavers via dye injection into the dorsal vein of the penis, an integral part of the prostatic venous plexus. Through these extensive injection studies of the prostatic plexus and venules of the breast in male and female cadavers, as well as in animals, Batson described this vertebral vein system as 1) consisting of the epidural veins, the perivertebral veins, the veins of the thoraco-abdominal wall, the veins of the head and neck, and the veins of the walls of blood vessels of the extremities; 2) valveless vessels that carry blood under low pressure; 3) subject to arrest and reversal of blood flow; 4) parallels, connects with, and provides bypasses for the portal, pulmonary, and caval systems. This plexus may serve as a major channel by which certain malignancies, such as prostate and breast cancer, metastasize to bone.

This concept that the vertebral system of veins acts as a direct conduit in the spread of prostatic carcinoma to the skeletal system was refuted by Dodds *et al.* (284), who analyzed ^{99m}technetium bone scans in patients with skeletal metastases from assorted primary tumors. They found that the distribution of metastases was virtually identical in patients with prostatic and nonprostatic tumors. Of the patients with prostatic carcinoma, 25% had bone scan lesions exclusively outside the region of the sacrum, pelvis, and lumbar spine. The distribution of skeletal metastases from prostatic carcinoma did not support the concept that the vertebral veins have a substantial role in the dissemination of this tumor.

2. Seed and soil. Regardless of whether or not blood flow or anatomic considerations are important determinants of the site of metastasis, they are not the only ones. The distribution of metastases to various organs are not predicted by anatomic considerations alone in approximately 40% of tumors (285). Thus, other determinants of the site of metastasis, such as properties of both the tumor cell and the metastatic site, are important. Metastasis is an extremely complex event that involves a cascade of linked sequential events that must be completed before a tumor cell successfully establishes a secondary tumor in bone (Fig. 5). Specifically, a tumor cell must 1) detach from the primary site; 2) enter tumor vasculature to reach the circulation; 3) survive host immune response and physical forces in the circulation; 4) arrest in distant capillary bed; 5) escape the capillary bed; and 6) proliferate in the metastatic site. The events involved in entering the tumor

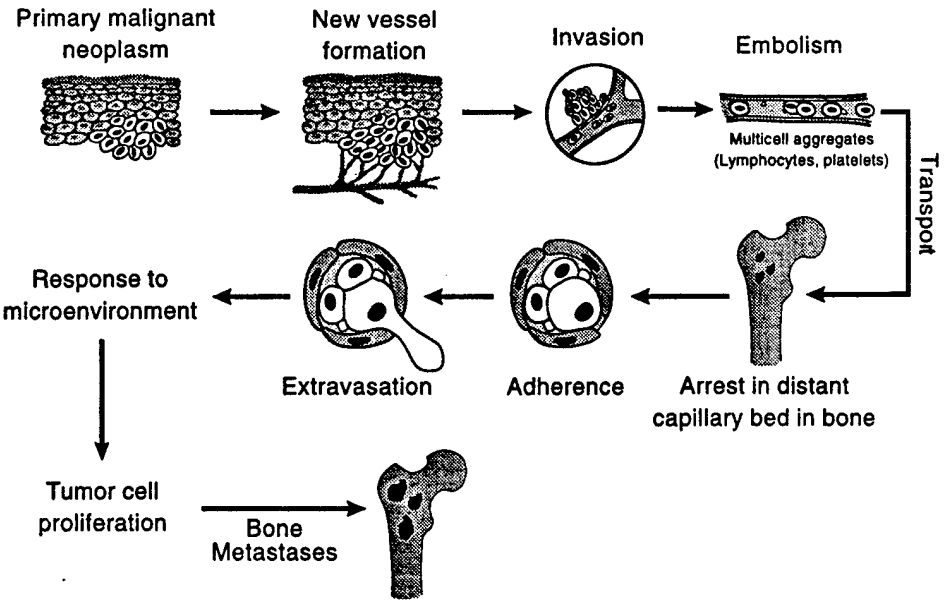

FIG. 5. General mechanisms of tumor cell metastasis to bone. Multiple steps involved in tumor cell metastasis from a primary site to the skeleton. Each of these steps represents a potential therapeutic target for the development of drugs to reverse or prevent metastatic bone disease.

vasculature are similar to those involved with exiting the vasculature in the bone marrow cavity. These include 1) attachment of tumor cells to the basement membrane; 2) tumor cell secretion of proteolytic enzymes to disrupt the basement membrane; and 3) migration of tumor cells through the basement membrane. Attachment of tumor cells to basement membranes and to other cells are mediated through cell adhesion molecules such as laminin and E-cadherin. Tumor cell secretion of substances such as metalloproteinases facilitate disruption of the basement membranes and enhance invasion. Inherent tumor cell motility or motility in response to chemotactic stimuli are also important factors for tumor cell invasion to the secondary site.

The fact that breast cancer is associated with significant morbidity in the skeleton was noted in 1889 when Stephen Paget observed that "in a cancer of the breast the bones suffer in a special way, which cannot be explained by any theory of embolism alone"(1). Indeed, breast cancer is one of a limited number of primary neoplasms that display osteotropism, an extraordinary affinity to grow in bone. Greater than 70% of women dying from breast cancer have bone metastasis (274–277). The mechanisms underlying this osteotropism are complex and involve unique characteristics of both the breast cancer cells and the bone to which these tumors metastasize.

Why is breast cancer one of the limited primary tumors to display osteotropism? Paget, during his observations of breast cancer in 1889, proposed the "seed and soil" hypothesis to explain this phenomenon. "When a plant goes to seed, its seeds are carried in all directions; but they can only grow if they fall on congenial soil" (1). In essence, the microenvironment of the organ to which the cancer cells metastasize may serve as a fertile soil on which the cancer cells (or seeds) may grow. Although this concept was proposed over a century ago, it remains a basic principle in the field of cancer metastasis at the present time. Thus, breast cancer cells possess certain properties that enable them to grow in bone, and the bone microenvironment provides a fertile soil on which to grow.

C. Local tumor syndromes in bone

Understanding the pathophysiology of bone metastasis has been a slow process scientifically, as very few useful animal models of spontaneous bone metastasis exist. Thus, various techniques of experimental bone metastasis have been used throughout the years and include injection of tumor cells directly into 1) the intramedullary cavity (286); 2) abdominal aorta (287); 3) tail vein with inferior vena cava occlusion (288); 4) left upper thigh muscle (289); 5) left thoracic artery with renal artery occlusion; and 6) left cardiac ventricle (290, 291). A complete review of the advantages and disadvantages of these models has been extensively discussed by Orr *et al.* (292).

1. Osteolytic metastases. Cancer metastatic to bone often causes bone destruction or osteolysis. Although several tumor types, such as prostate, lung, renal cell, and thyroid, are associated with osteolytic lesions, breast cancer is the most common. A comprehensive review of more than 500 patients dying of breast cancer revealed that 69% had bone metastasis, and bone was the most common site of first distant relapse (182). In those patients with disease confined to the skeleton, the median survival was 24 months compared with 3 months in those patients whose first relapse occurred in the liver. For these reasons, the following discussion on the pathophysiology of cancer-mediated osteolysis will focus on breast cancer.

a. Breast cancer cells as the seed: A variety of common characteristics are necessary for tumor cells to possess the metastatic phenotype. Such properties include 1) the production of proteolytic enzymes necessary for detachment from the primary site, invasion into surrounding soft tissues, intravasation, extravasation, and bone matrix degradation; 2) expression or loss of cell adhesion molecules essential for detachment from the primary site and arrest at a metastatic site; 3) migratory activity to travel in the circulation; 4) escape from the host immune surveillance to survive; and 5) capacity to respond to a chemoattractant. Although these properties are common to tumor cells metastasizing to any organ, they are insufficient to explain the propensity of breast cancer to metastasize to bone. Therefore, it is likely that breast cancer cells have additional characteristics that are specifically required for causing metastases in bone.

Since bone is mainly composed of a hard mineralized tissue, it is more resistant to destruction than other soft tissues. Thus, in order for cancer cells to grow in bone, they must possess the capacity to cause bone destruction. Histological review of breast cancer metastatic to bone reveals that tumor cells are adjacent to osteoclasts resorbing bones (184–186) and indicate that breast cancer cells possess the capacity to stimulate osteoclastic bone resorption. Breast cancer cells may either induce osteoclastic differentiation of hematopoietic stem cells, activate mature osteoclasts already present in bone, or do both, through releasing soluble mediators or via cell-to-cell contact. Clinical and experimental evidence indicates that tumor-produced PTHrP is a major candidate factor responsible for the osteoclastic bone resorption present at sites of breast cancer metastatic to bone (293–295). PTHrP has been detected by immunohistochemistry (293) and *in situ* hybridization (294) in 92% of breast cancer metastases in bone compared with only 17% of similar metastases to non-bone sites, an observation that prompted speculation that production of PTHrP as a bone-resorbing agent may contribute to the ability of breast cancers to grow as bone metastases. Bundred and colleagues (195) found positive immunohistochemical staining for PTHrP in 56% of 155 primary breast tumors from normocalcemic women, and PTHrP expression was positively correlated to the development of bone metastases and hypercalcemic episodes. PTHrP expression was detected by RT-PCR in 37 of 38 primary breast cancers, and subsequent development of bone metastases was associated with a higher PTHrP expression (295). Finally, PTHrP was detected by immunohistochemistry in 83% of patients who developed bone metastases compared with 38% in those who developed lung metastases and 38% in those without recurrence (196). There have been no consistent correlations between PTHrP expression in the primary breast tumor and standard prognostic factors, recurrence, or survival. The only significant and consistent

correlations have been between PTHrP positivity and the development of bone metastases and hypercalcemia.

These clinical observations have been extended by using a mouse model of bone metastases (290, 296) in which inoculation of a human breast cancer cell line, MDA-MB-231 (297), into the left cardiac ventricle reliably causes osteolytic metastases. MDA-MB-231 cells produce low amounts of PTHrP *in vitro* and when the cells were engineered to overexpress PTHrP, by transfection with the cDNA for human prepro-PTHrP, an increase in the number of osteolytic metastases was observed (178). In contrast, when mice were treated with monoclonal antibodies directed against the 1–34 region of PTHrP, before inoculation with parental MDA-MB-231 cells, the number and size of observed osteolytic lesions were dramatically less than similar animals treated with control (Fig. 6). Mice with established osteolytic metastases due to MDA-MB-231, treated with the antibody, had a decrease in the rate of progression of metastases when compared with mice that received a control injection (282, 298). Similar findings have been demonstrated in this model using a human lung squamous cell carcinoma (299). Taken together, these data strongly suggest that PTHrP expression by breast cancer cells is important for the development and progression of breast cancer metastases in bone. It stands to reason, then, that production of other osteoclast-stimulating factors should potentiate the development of bone metastases as well.

Just as production of bone-resorbing factors by breast cancer cells enhances bone metastases, production of other factors may render the breast cancer cell ineffective as a seed and result in less metastases in bone or other sites. Using the same mouse model of breast cancer metastases to bone, MDA-MB-231 cells transfected to overexpress either the cell adhesion molecule, E-cadherin, or tissue inhibitor of metalloproteinase-2 (TIMP-2) inoculated into nude mice resulted in a decrease in osteolytic metastases compared with nontransfected MDA-MB-231 cells (300, 301).

Cancer cell expression of factors affecting motility are important in the general metastatic process (302) as well in those processes specific to bone metastasis. Once tumor cells arrive in the bone marrow sinusoids, they must possess the capacity to move through those sinusoids to the bone tissue. Autocrine motility factor (303, 304), Thymosin β15, and possibly the small heat shock protein 27 (Hsp27) have emerged as potential factors controlling cell motility. Thymosin β15 increases cell motility, and when its production was decreased by expression of antisense constructs, as recently reported by Bao *et al.* (305), metastases were prevented in the Dunning rat prostate adenocarcinoma model. Similarly, overexpression of Hsp27 in MDA-MB-231 cells decreased cell motility *in vitro* and bone metastasis in mice (306).

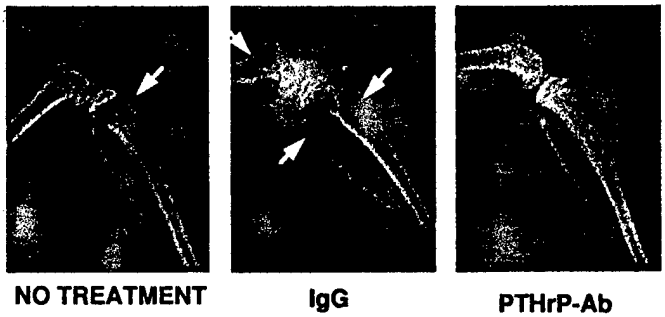

FIG. 6. Radiographs of osteolytic bone lesions in hind limbs from female nude mice inoculated via the left cardiac ventricle from respective treatment groups of nothing, control IgG, and PTHrP antibody. Radiographs were taken 26 days after tumor inoculation with MDA-MB-231 cells. Arrows indicate osteolytic metastases in distal femur, proximal tibia, and fibula. [Reproduced from T. A. Guise *et al.* (282) by copyright permission of The American Society for Clinical Investigation, Inc.]

Another important property of the breast cancer seed that enables it to establish growth in bone resides in the adhesion molecules. Experimental evidence supports the notion that tumor cell surface expression of such molecules mediates targeting to bone and the resultant development of bone metastasis. For example, bone marrow stromal cells express the vascular cell adhesion molecule-1 (VCAM-1), a ligand for $\alpha_4\beta_1$ integrin (307). Tumor cells expressing $\alpha_4\beta_1$ integrin may preferentially adhere to bone marrow stromal cells to establish bone metastasis. CHO cells transfected with $\alpha_4\beta_1$ caused bone and lung metastases when inoculated intravenously into nude mice compared with only lung metastases in mice similarly inoculated with untransfected CHO cells (308). In that report, bone metastases were inhibited by antibodies against $\alpha_4\beta_1$ or VCAM-1. Similar expression of $\alpha_3\beta_1$, $\alpha_6\beta_1$, or $\alpha_v\beta_1$ did not induce bone metastases (308). Although many breast cancer cells express the $\alpha_v\beta_3$ integrin receptor that binds the bone matrix protein, osteopontin, a potential avenue for the development of bone metastasis, MDA-MB-231 cell populations with high-level expression of the $\alpha_v\beta_3$ integrin were less likely to cause bone metastasis than those cells expressing low amounts of $\alpha_v\beta_3$ in the mouse model of bone metastasis (309). Bone sialoprotein peptides containing RGD sequences have been shown to decrease MDA-MB-231 cell adhesion to extracellular bone matrix *in vitro* (310). Finally, tumor cell expression of CD44 may mediate binding to osteopontin via RGD-independent mechanisms (311). Such observations illustrate the complex and multifactorial nature of the mechanisms underlying the metastatic process.

Taken together, tumor cell expression of osteolytic factors, adhesion molecules, and motility factors significantly impact the ability of the tumor cell, or seed, to develop and grow as bone metastases.

b. Bone microenvironment as the soil: Bone is unique among metastatic target tissues since it undergoes continual remodeling under the influence of systemic hormones and local bone-derived growth factors. Mineralized bone matrix is a repository for growth factors, of which TGFβ and IGF-II constitute the majority (3). As described earlier, these growth factors are released from the bone matrix as a result of normal osteoclastic bone resorption (5), a part of the normal remodeling process necessary for maintenance of the structural integrity of bone. The hematopoietic stem cells in the bone marrow can differentiate into bone-resorbing osteoclasts. Other cells in the bone marrow, stromal and immune cells in particular, produce cytokines and growth factors that may potentiate tumor cell growth or expression of osteolytic factors. Thus, once breast cancer cells arrest in bone, the high concentrations of growth factors and cytokines in the bone microenvironment provide a fertile soil on which the cells

can grow. Such host cytokines may also enhance osteoclastic bone resorption stimulated by tumor-produced factors such as PTHrP. Furthermore, when the tumor cells stimulate osteoclastic bone resorption, this bone microenvironment is even more enriched with bone-derived growth factors that enhance survival of the cancer. Finally, bone-derived TGFβ may have an important role as a chemoattractant for breast cancer cells.

A large body of indirect evidence to support the concept that bone is a fertile soil, further enriched by the process of osteoclastic bone resorption, has accumulated in studies using bisphosphonates in the treatment of bone metastases. It is already clear from clinical studies that the use of bisphosphonates, potent inhibitors of bone resorption, significantly reduces skeletal morbidity in advanced breast cancer (312–323). In a recent multicenter trial that consisted of more than 700 patients with stage IV breast cancer with two or more predominantly lytic lesions, with at least one lesion that was 1 cm or greater in diameter, treatment with pamidronate 90 mg iv every 3–4 weeks for 12 months in conjunction with chemotherapy or hormonal therapy resulted in a significant reduction in skeletal complications and bone pain compared with the control group (321). Bisphosphonates have also been shown to decrease the number of bone metastases as well as tumor burden in animal models (296, 324, 325). Thus, by decreasing osteoclastic bone resorption, the bone microenvironment is a less fertile soil for the growth of tumor.

TGFβ, which is present in high concentrations in the bone microenvironment and expressed by some breast cancers (177, 178) and cancer-associated stromal cells (176), has been shown to enhance secretion of and stabilize the mRNA for PTHrP in a renal cell carcinoma (96), a squamous cell carcinoma (97, 98), and a human breast adenocarcinoma, MDA-MB-231 (177, 178). In fact, of the known growth factors present in the mineralized bone matrix other than TGFβ, such as IGF-I and -II, fibroblast growth factors (FGFs) 1 and 2, bone morphogenetic proteins (BMPs) and platelet-derived growth factor, only TGFβ has been shown to significantly stimulate PTHrP secretion from the human breast cancer cell line, MDA-MB-231 (178). The fact that TGFβ is abundant in bone (3) and can enhance PTHrP expression by cancer cells makes it an important candidate factor in the establishment and progression of breast cancer metastases to bone. TGFβ is a member of a large superfamily of proteins that are important regulators of bone cell activity (326). Multiple isoforms of TGFβ exist in mammals and appear to control cell proliferation and differentiation in many human cell types (327). The prototype of these isoforms, TGFβ1, is highly expressed by differentiated osteoblasts and osteoclasts, is stored in bone matrix, and is released in active form during osteoclastic bone resorption (5). The effects of TGFβ include stimulation of cell proliferation of mesenchymal cells, growth inhibition of epithelial cells, synthesis of extracellular matrix proteins, and enhancement of cell adhesion. These effects of TGFβ are mediated through complex receptor interactions (328). TGFβ binds to the type II receptor, and this complex recruits and phosphorylates the type I receptor, which in turn initiates signal transduction mediated by the recently identified Smad protein family (328). The effects of TGFβ on cancer cells are complex and variable (329). In some cancer cells, TGFβ inhibits growth, while in others growth is stimulated (330). It likely has effects on apoptosis as well.

Further evidence for the role of bone-derived TGFβ in the development and progression of breast cancer metastasis to bone has been demonstrated in the same in vivo animal model of osteolysis described above. Since TGFβ increases PTHrP expression by MDA-MB-231 cells *in vitro*, this cell line was transfected with a cDNA encoding a TGFβ type II receptor lacking a cytoplasmic domain (TβRIIΔcyt) (331). This receptor binds TGFβ, but since it cannot phosphorylate the type I receptor, signal transduction is not initiated and it acts in a dominant-negative fashion to block the biological effects of TGFβ (332). Stable clones expressing TβRIIΔcyt did not increase PTHrP secretion in response to TGFβ stimulation compared with controls of untransfected MDA-MB-231 cells or those transfected with the empty vector. Mice inoculated into the left cardiac ventricle with MDA-MB-231 cells expressing TβRIIΔcyt had fewer osteolytic lesions as well as a smaller area of osteolytic lesions by radiography and histomorphometry compared with the controls of parental cells or those transfected with the empty vector (333). These data indicate that TGFβ responsiveness of this human breast cancer cell line is important for the expression of PTHrP in bone and the development of osteolytic bone metastasis *in vivo*.

In the mouse model of bone metastasis in which human tumor cells inoculated into the left cardiac ventricle cause bone metastasis, metastasis to the calvariae rarely occur due to the relatively low rate of bone turnover at this site compared with other bones. To increase the rate of bone turnover in the calvariae, Sasaki *et al.* (334) injected recombinant IL-1α subcutaneously over the calvariae of nude mice for 3 days. Upon completion of these treatments, MDA-MB-231 cells were inoculated into the left cardiac ventricle of female nude mice. Four weeks after tumor cell inoculation, IL-1-treated mice had obvious metastatic tumor deposits in the calvariae compared with none in the control-treated mice. Further experiments demonstrated that pretreatment of the mice injected with IL-1 with the bisphosphonate, risedronate, profoundly diminished the development of metastatic tumor deposits in the calvariae (334). These data suggest that growth factors released from bone matrix may potentiate tumor cell growth in bone metastases. However, these findings do not exclude some other alteration in the bone matrix or microenvironment that may be enhanced due to increased bone turnover.

Growth factors released from resorbing bone likely have significant effects on tumor cell growth as well. Experimental evidence suggest that IGFs may be important in this regard. Culture supernatants from resorbing neonatal mouse calvariae strongly increased the proliferation of MDA-MB-231 breast cancer cells in culture (335). Inhibition of bone resorption by adding risedronate to the calvarial organ cultures blocked the subsequent breast cancer cell proliferation. Additionally, neutralizing antibodies to the IGF-I receptor markedly impaired the growth-stimulating effects of the resorbing bone culture supernatants on the tumor cells (335). These results strongly suggest that IGFs are released from bone during bone resorption and promote breast cancer cell proliferation.

Another property of bone that may explain the predilec-

tion of certain tumor types to grow in bone is chemotactic attraction of circulating cancer cells. Culture supernatants of resorbing bone stimulate chemotactic movement of breast cancer cells in a Boyden chamber assay (336). Bone matrix factors such as TGFβ, type I collagen and its fragments, osteocalcin, and IGFs have been shown to stimulate chemotaxis of breast cancer cells (337). Most recently, IGF-I was shown to stimulate $\alpha_v\beta_5$ integrin-mediated chemotactic migration of human breast cancer cell lines (338).

c. Tumor cell-bone interactions: In addition to the properties of breast cancer as a seed and the bone microenvironment as a soil, there are likely complex interactions between the bone microenvironment and the tumor cell as well as between and within tumor cells that influence osteoclast activation. Both bone-derived and tumor-associated factors have been shown to increase PTHrP expression by tumor cells as well as modulate the end-organ effects of PTHrP. Thus, such factors may enhance the ability of tumor cells to activate osteoclasts and promote bone destruction.

Other tumor-associated factors in addition to bone-derived growth factors may be important regulators of PTHrP expression in breast cancer metastatic to bone. As indicated in the previous section on hypercalcemia, many tumor-associated factors, such as EGF (90, 91), TGFα (166), IL-6 (165), TNF, IGF-I, and IGF-II (91), have the potential not only to enhance tumor production of PTHrP but to modulate its end-organ effects on bone as well.

d. Implications of PTHrP status in breast cancer: These findings have important implications for breast cancer effects on the skeleton. First, breast cancers expressing PTHrP may affect the skeleton through humoral and osteolytic mechanisms. Second, the effects of PTHrP on bone may be enhanced if the breast cancer expresses other bone-active factors, such as TGFα or IL-6, in addition to PTHrP. Finally, growth of breast cancer cells in bone may be enhanced if the tumor cells express PTHrP or other bone-resorbing factors. TGFβ, as well as other bone-derived growth factors, increase PTHrP expression by breast cancer cells in bone, so that TGFβ-responsive tumors may preferentially grow in bone. Thus, enhanced osteoclastic bone resorption causes increased release of TGFβ and other growth factors into the bone microenvironment. The result is 1) greater PTHrP expression by the breast cancer cells; 2) enhanced growth of the cancer cells; and 3) chemoattraction of more tumor cells by bone-derived factors. A cycle is thus established, as illustrated in Fig. 7, which ends in bone destruction and the other consequences of lytic bone metastases. The clinical finding of increased PTHrP expression in bone compared with other sites supports the notion that production of PTHrP as a bone-resorbing agent may contribute to the ability of breast cancers to grow as bone metastases and/or that the bone microenvironment enhances production of PTHrP. These and other reasons may, in part, explain the propensity of breast cancers to metastasize to bone and the alacrity with which breast cancer grows in bone.

If PTHrP expression in the primary breast tumor indicates a propensity to metastasize and destroy bone due to its potent bone-resorbing capability, early treatment with inhibitors of bone resorption or agents that inhibit the production of or biological effects of PTHrP are likely to prevent or delay the development of bone metastases as well as reduce the catastrophic complications of pain, hypercalcemia, fracture, and nerve compression syndromes.

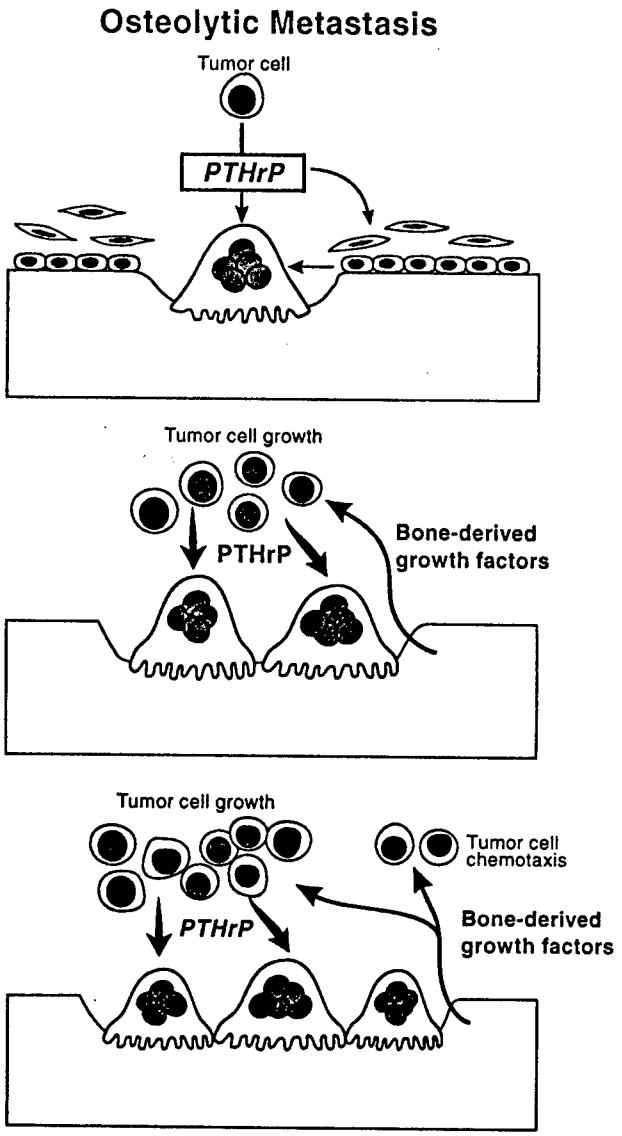

FIG. 7. Schematic illustration of a proposed mechanism of local bone destruction in osteolytic bone metastasis mediated by PTHrP. Other osteolytic factors may mediate this process as well. In the *top panel*, tumor cell arrives in bone and stimulates osteoclastic bone resorption via secretion of PTHrP, an effect that is mediated through the osteoblast and stromal cells. In the *middle panel*, osteoclastic bone resorption results in release and activation of growth factors present in bone matrix, such as TGFβ, IGF-I and -II, etc. Such factors may increase tumor production of PTHrP (in the case of TGFβ) and/or increase tumor cell growth (in the case of IGFs). The *lower panel* illustrates the end result of this cycle in which increased tumor-stimulated osteoclastic bone resorption results in increased local concentration of bone-derived growth factors. Such factors increase PTHrP production, tumor cell growth, and chemotaxis.

2. *Osteoblastic metastasis.* Osteoblastic metastases occur most commonly in prostate cancer and less so in breast cancer. Rarely, osteoblastic bone lesions have been described in other malignancies such as an osteosclerotic variant of myeloma (279), colon cancer (339), astrocytoma (340), glioblastoma

multiforme (341), thymoma (342), carcinoid (343), nasopharyngeal carcinoma (344), leptomeningial gliomatosis (345), Zollinger-Ellison syndrome (346), and cervical carcinoma (347). Similar to the pathophysiology of breast cancer-mediated osteolysis, the seed and soil hypothesis applies to this situation as well in that tumor cells secrete factors that stimulate bone formation, and the bone microenvironment readily supports the growth of prostate cancer cells (348).

Prostate cancer is relatively unique in its ability to form osteoblastic bone metastases, and there is much speculation on the mechanisms involved. Understanding the pathophysiology of prostate cancer-mediated osteoblastic metastasis has been limited, in part, to the paucity of *in vivo* models that adequately reproduce the spectrum of human disease, including the osteoblastic phenotype. However, over the past few years several models have been reported that may provide insight into the mechanisms responsible for the osteoblastic metastasis. Thalmann *et al.* (349) reported that an androgen-independent clone of the LNCaP human prostate cancer cell line, when inoculated subcutaneously or orthotopically into castrated male nude mice, spontaneously metastasized to bone in 11–50% of mice. Histological evidence of new bone formation was observed at the site of bone metastasis. Greenberg *et al.* (350) developed a transgenic mouse model of spontaneous prostate cancer, in which the simian virus 40 (SV40) large tumor T antigen is driven by the rat probasin promoter to target the dorsolateral epithelium of the prostate (TRAMP mice). In this model, 100% of mice develop distinct pathology in the dorsolateral epithelium of the prostate by 10 weeks of age that range from mild intraepithelial hyperplasia to large multinodular malignant neoplasia (350). Distant metastases occur as early as 12 weeks in common sites of periaortic lymph nodes and lungs and less common sites of kidney, adrenal gland, and bone (351). As reported by Gingrich *et al.* (351), one of these TRAMP mice developed paraplegia and was found to have tumor metastatic to the spinal canal. Bone pathology revealed osteoclastic bone resorption and new bone formation at vertebral sites adjacent to the spinal metastasis. Finally, in a model similar to the one described previously for breast cancer metastasis to bone, described in a later section of this review, rat prostate cancer cells inoculated into the left cardiac ventricle of syngeneic rats caused osteoblastic bone metastasis (352). Further investigation of these animal models should provide significant insight into the pathophysiology of prostate cancer metastasis to bone.

a. Prostate cancer as the seed: As prostate cancer is more frequently associated with osteoblastic metastases, prostate cancer cells must possess properties different from those of other tumor types commonly associated with osteolytic metastases. The histomorphometric studies of Charhon et al. (353) indicate that osteoblastic metastases are likely due to soluble factors that are produced by metastatic prostate cancer cells that stimulate bone formation. Osteoblast-stimulating activity produced by prostate cancer has been described by a number of investigators. Conditioned media from *Xenopus* oocytes injected with total RNA from the human prostate cancer cell line PC3 stimulated both mitogenesis and alkaline phosphatase activity in osteosarcoma cells with the osteoblast phenotype (354). In fetal rat calvarial cells, PC-3-conditioned media stimulated osteoblast proliferation (355). Koutsilieris *et al.* (356) found that extracts of prostate cancer tissue and normal prostate tissue stimulated proliferation of bone cells.

b. Osteoblastic factors: Such data indicate that prostate cancer is a source of osteoblast-stimulating activity. The following tumor products have been proposed to be important in the genesis of the osteoblastic response to tumor cells in bone.

i. TGFβ. TGFβ is secreted by osteoblasts in a latent biologically inactive form that is incorporated into bone extracellular matrix. TGFβ is synthesized as latent high molecular mass complexes, composed of TGFβ, the amino-terminal portion of the TGFβ precursor, and the latent TGFβ-binding protein (LTBP). Osteoblasts not only produce TGFβ but they also possess high-affinity receptors for it (357), providing the opportunity for autocrine stimulation of osteoblast replication. Latent TGFβ can be activated by a number of agents including acid pH, or by proteases such as plasmin or cathepsin D (358, 359). TGFβ1 and -2 are homologous disulfide-linked homodimers of 25 kDa that have powerful effects on bone.

The local function of TGFβ may be very important in contributing to the differentiated activity of osteoblasts. It stimulates collagen synthesis and regulates gene expression of mRNA for pro-αI (I) collagen, osteonectin, alkaline phosphatase, fibronectin (360), osteopontin (361), and osteocalcin (362). TGFβ increases the abundance of matrix proteins by stimulating their synthesis and inhibiting their degradation. It is a potent stimulator of collagen and fibronectin synthesis and secretion in fibroblasts and osteoblasts (363, 364), acting by increasing the mRNA for collagen and fibronectin (365). TGFβ also inhibits degradation of matrix proteins by decreasing the synthesis of matrix-degrading enzymes, as well as increasing the synthesis of protease inhibitors (366). TGFβ promotes the differentiation of cells of the osteoblast lineage toward the mature osteoblast and the formation of new bone.

TGFβ, injected subcutaneously adjacent to bone surfaces, causes a profound increase in new bone formation (367–369). When TGFβ is administered by injection over the calvariae of mice daily for 3 days, bone width is increased 40% over the next month (368). This is initially woven bone, but it is later replaced by lamellar bone. Similar effects are seen when TGFβ is injected or infused directly into the marrow cavity of the femur.

TGFβ may also affect osteoclastic bone resorption as indicated by recent studies using a transgenic mouse model in which active TGFβ2 overexpression was targeted to osteoblasts through the use of an osteocalcin promoter (370). This osteoblast-specific overexpression of TGFβ2 resulted in progressive bone loss associated with increases in osteoblastic matrix deposition and osteoclastic bone resorption.

TGFβ, isoforms 1 and 2 in particular, are produced by prostate cancer. TGFβ2 is produced in abundant amounts in the human prostate cancer cell line PC3 (371). Although most studies in primary prostate indicate that TGFβ is produced by prostate cancer cells, one study demonstrated immunohistochemical localization of TGFβ in peritumoral fibroblasts (372). TGFβ expression in human prostate cancer tissue appears to be greater than in normal prostate or benign prostatic hypertrophy (373). Studies from histopathologically

verified human prostate cancer indicate that TGFβ is produced without associating with the LTBP whereas in normal and benign prostatic hyperplasia tissues, TGFβ may be produced in a complex associated with LTBP (374). Other investigators have found that prostate cancer cells secrete TGFβ1 in the latent form, and the prostate cancer cells themselves further activate approximately 50% to the bioactive form (373). Other studies have shown that overexpression of TGFβ1 in the rat prostate cancer cell line MATLyLu was associated with enhanced growth, viability, and aggressiveness *in vivo* (375). Although there is little evidence in the literature to demonstrate a direct relationship between tumor-produced TGFβ and the development of osteoblastic metastasis, the fact that TGFβ is produced by human prostate cancer coupled with its profound effects on bone formation have obvious implications in the pathophysiology of prostate cancer-mediated osteoblastic metastasis.

ii. IGF-I and -II. The IGF system is a fairly complex one and consists of two ligands, IGF-I and IGF-II, two receptors, and six binding proteins. The topic of IGFs, their binding proteins, and their biological actions has been extensively reviewed by Jones and Clemmons (376). Most of the cellular effects of the IGFs are mediated by binding of the peptides to the IGF-I receptor. The affinity of the IGF-I receptor for IGF-II is 2- to 15-fold lower than for IGF-I. The IGF-II/cation-independent mannose 6-phosphate receptor binds IGF-II with a 500-fold greater affinity than IGF-I. IGFs mediate their biological effects via interaction with their respective receptors, and these receptor interactions are affected by the presence of IGF-binding proteins. Six IGF-binding proteins (IGFBPs) have been identified, and binding of these proteins to IGFs can enhance or inhibit the biological effects of IGFs. IGF-I and -II are weak bone cell mitogens, but have clear and potent stimulatory effects on the differentiated function of the osteoblast, as evidenced by an increase in osteocalcin and type I collagen synthesis in osteoblasts. As a result, IGFs increase bone matrix apposition rates and bone formation. IGFs also decrease collagen degradation and the expression of interstitial collagenase, functions that suggest a role in the preservation of bone matrix. IGFs enhance bone formation *in vivo*, and mice with null mutation of type I IGF receptor have delayed skeletal development and ossification (377). The anabolic properties of IGF-I and -II, their inhibitory actions on matrix degradation, and their abundance in bone tissue suggest that these factors play a central role in the maintenance of bone mass (378).

Regulation of IGF-I in bone is further complicated by the production of IGFBPs by osteoblasts, which express all six IGFBPs. Binding of IGF to one of these binding proteins can inhibit or potentiate the biological effect of IGF. Binding to IGFBP-1, for example, decreases the biological activity of IGF-I. Conversely, IGFBP-5 has been shown to increase bone formation and, thus, appears to enhance the effect of IGF-I. This system is further complicated by the observation that growth factors such as TGFβ, platelet-derived growth factor, FGF, and BMP-2 inhibit synthesis of IGFBP-5 in bone cell cultures (379, 380) while IGF-I and retinoic acid increase it (381). Thus, it appears that the effect of IGFs on bone is anabolic, and that local regulation of IGFs in bone is highly complex.

IGFs are potent mitogens for the growth of human prostate cancer cells, and primary cultures of prostate epithelial cells have been demonstrated to express all aspects of a functional IGF system: IGFs, IGF receptors, and IGFBPs (382). Human seminal fluid contains IGF-I and -II, IGFBP-2 and -4, as well as IGFBP-3 fragments and IGFBP-3 protease activity (383). This IGFBP-3 protease activity in seminal fluid has been attributed to prostate-specific antigen (PSA) (384) while production of other proteases such as urokinase receptor and cathepsin D have been demonstrated in prostate cancer (385, 386). IGFBP-2 appears to be the main binding protein produced by prostate cancer cells and, accordingly, clinical studies have demonstrated serum concentrations of IGFBP-2 to be increased and IGFBP-3 to be decreased in patients with prostate cancer (387, 388). Furthermore, significant positive correlations between serum concentrations of IGFBP-2 and PSA as well as between IGFBP-2 and tumor stage have been observed in men with prostate cancer (387, 388). Although at least one of these studies included patients with bone metastases, neither report comments on whether there was a significant correlation between IGFBP-2, PSA, and the presence of bone metastases. Immunohistochemistry and *in situ* hybridization in prostate tissue containing benign epithelium, high-grade prostate intraepithelial neoplasia, and adenocarcinoma indicate that mRNA and immunostaining intensity for IGFBP-2 progressively increased from benign prostate tissue to malignant adenocarcinoma whereas the immunostaining intensity for IGFBP-3 was increased in prostate intraepithelial neoplasia compared with normal, but decreased in malignant, cells (389). These authors conclude that the decreased expression of IGFBP-3 in malignant prostate tissue may be due to pre- and/or posttranslational mechanisms, including proteolysis, and that these observations correlate with serum changes of IGFBPs described in men with prostate cancer.

There is accumulating evidence that prostate cancers produce a variety of proteases, such as PSA, urokinase type plasminogen activator, and cathepsin D, that may be responsible for dissociating IGF-I and IGF-II from respective binding proteins to result in enhanced effects on not only tumor growth, but also, in the case of prostate cancer metastatic to bone, mitogenic effects on osteoblasts. PAIII cell-conditioned media has been shown to contain a 35-kDa proteinase capable of digesting IGFBPs that may serve to increase the bioavailability of osteoblast-derived IGFs (390). In addition to these proteolytic effects to activate growth factors, these proteases may be mitogenic for tumor cells as well.

Based on the above observations of the presence of an intact IGF system (including IGFBP proteases) in prostate cancer, the mitogenic effect of IGFs on prostate cancer, as well as on osteoblasts, and the positive correlations between serum IGFBP-2 and PSA, it is conceivable that local production of IGFs by prostate cancer in bone may mediate the osteoblastic response so characteristic of prostate cancer metastatic to bone. Unfortunately, the data described above are associations at best, and a direct causal role has yet to be proven.

iii. Proteases.

1. PSA. PSA is a serine protease, single-chain glycoprotein

that has trypsin-like and chymotrypsin-like enzymatic activity (391). As PSA was initially believed to be produced exclusively by prostate epithelial cells, it has been extensively used as a marker for prostate cancer (392). The three clinical diseases associated with an increased serum PSA concentration are prostate cancer, benign prostatic hypertrophy, and acute bacterial prostatitis (393). In patients with prostate cancer, the serum PSA concentration is a valuable biological marker for diagnosis, prognosis, and management. The pretreatment serum PSA concentration has been shown to be a significant predictor of disease outcome after radiation therapy for local and regional prostate cancer (394). Androgenic hormones increase the production of PSA via transcriptional regulation (395). Serum PSA concentrations have been shown to correlate significantly with the presence of bone metastases by radionuclide scanning (396). In a large clinical study of 521 men with newly diagnosed and untreated prostate cancer, only one of 306 patients with a serum PSA concentration of less than 20 ng/ml had a positive bone scan (397). Serum PSA concentration proved to be the best predictor of bone scan findings when compared with tumor grade, local clinical stage, acid phosphatase, and prostatic acid phosphatase (397–399). Thus, in a newly diagnosed patient with prostate cancer, a serum PSA concentration of less than 10 ng/ml, and no skeletal symptoms, a bone scan may not be necessary (396) although others recommend measurement of PSA in conjunction with bone-specific alkaline phosphatase (400). Immunoreactive PSA has recently been demonstrated in 27% of 174 primary breast cancers (401) even though it was once believed to be an exclusive product of prostate epithelium. Breast-derived PSA was identical to PSA derived from prostate (402), and PSA has been shown to be produced at the ovarian metastatic site of a breast cancer (403). Furthermore, in a larger study of breast tumor cytosols from women and men, a positive correlation between immunoreactive PSA and progesterone receptor was observed (404).

The function of PSA in prostate cancer is unclear, but its proteolytic activity may prove to be important in the genesis of osteoblastic response to prostate tumor in bone. PSA has been shown to proteolyze IGFBP-3 into at least seven fragments with molecular masses of 13 kDa to 26 kDa with at least five different proteolytic recognition sites in this binding protein for PSA (405). Three of the five proteolytic sites were consistent with a kallikrein-like enzymatic activity while two of the sites were consistent with a chymotryptic-like enzymatic activity. Furthermore, some of the IGFBP-3 fragments retained the ability to bind IGF (405). Additionally, PSA has been shown to stimulate osteoblast proliferation at concentrations of 2.5 ng/ml possibly through activation of latent TGFβ (406). Thus, it is tempting to speculate that PSA-induced proteolytic cleavage of IGFBP-IGF complex results in locally active IGF at the site of prostate cancer metastatic to bone to stimulate the osteoblastic response. Furthermore, recent evidence demonstrates that PSA also cleaves PTHrP-(1–141) at the carboxyl-terminal phenylalanine 23 and inactivates the biological effects of PTHrP to stimulate cAMP production in an osteoblast cell line (407). This may have important implications for the predominantly osteoblastic phenotype observed in prostate cancer. The fact that breast cancers also express PSA is equally interesting. Metastatic breast cancer to bone is one of the few other carcinomas associated with osteoblastic metastases, albeit at a much lower frequency than observed with prostate cancer.

2. *Urokinase type plasminogen activator (uPA).* Urokinase-type plasminogen activator is a member of the serine protease family that also includes tissue-type plasminogen activator (tPA). These proteins are expressed in normal cells, and the major function of tPA is related to intravascular thrombolysis while uPA is involved in proteolysis during cell migration and tissue remodeling. Although both tPA and uPA have been identified in malignant tissue, uPA appears to have a more prominent role in malignancy by promoting tumor cell migration and invasion by activating plasminogen to plasmin which, in turn, cleaves extracellular matrix components of laminin, fibronectin, and collagen.

uPA has been isolated from several prostate cancer cell lines that promote new bone formation *in vivo*. The rat prostate PA III tumor line causes new bone formation when inoculated over the scapula of rats and athymic nude mice (408). Conditioned media from PA III cells stimulated proliferation of osteoblasts *in vitro*. uPa expression by human PC-3 prostate cancer cells is increased by EGF and *trans*-retinoic acid and decreased by dexamethasone (409). In an experiment to demonstrate the influence of uPA on the nature of prostate cancer metastasis, Achbarou *et al.* (352) used gene transfer techniques to overexpress uPA in the rat prostate cancer cell line, Mat LyLu, by 5-fold compared with the same cells expressing empty vector. A separate Mat LyLu cell line that expressed uPA mRNA in the antisense orientation had 3-fold reduction in uPA mRNA compared with the empty vector cells. The uPA-overexpressing, underexpressing, and parental cell lines were compared in a rat model of bone metastases in which tumor cells inoculated into the left cardiac ventricle of inbred male Copenhagen rats cause bone metastasis. Rats inoculated with the uPA-overexpressing cell line developed hind limb paralysis sooner than rats inoculated with empty vector Mat LyLu cells. Similarly, rats inoculated with the uPA antisense-expressing Mat LyLu cells developed hind limb paralysis later that rats inoculated with parental or uPA-overexpressing Mat LyLu (352). Histological assessment of the sites of tumor metastasis indicated that more metastatic tumor was present sooner in both skeletal and nonskeletal sites of the rats inoculated with the uPA-overexpressing Mat LyLu cell line, compared with those inoculated with the empty vector or antisense cell line. Furthermore, histological analysis of bone indicated that although both osteolytic and osteoblastic lesions were present in both control and experimental rats, the osteoblastic response was the predominant feature in rats bearing the uPA-overexpressing Mat LyLu cells.

iv. FGFs. Both acidic and basic FGFs, now known as FGF-1 and -2, respectively, are present in mineralized bone matrix and stimulate the replication of cells in the skeletal system, but do not increase the differentiated function of the osteoblast. Therefore, they may play an important role in bone repair where bone cell mitogenesis may be necessary (378). FGFs enhance TGFβ expression in cells with the osteoblast phenotype and have powerful stimulatory effects on bone formation *in vivo*. When injected locally over the calvariae of mice, FGF causes a 50% increase in bone thickness. When administered to ovariectomized rats, FGF blocked the asso-

ciated bone loss and also increased trabecular connectivity and bone microarchitecture (410).

Prostate cancer cells express large amounts of both FGF-1 and -2 (411, 412). Not only have various prostate cancer cell lines been demonstrated to produce FGF-1 and -2 (413–415) as well as FGF receptor, but other tumor-produced FGF-like polypeptides have been demonstrated as well (415). An extended amino-terminal form of FGF-2 was purified from a human amnion tumor by its ability to stimulate proliferation of the osteoblast cell line MG-63 (416). This tumor has been reported to cause bone formation *in vivo* when inoculated into nude mice. Other data suggest that FGF-2 inhibits osteoclast formation via stromal cells and osteoblasts (417). Although this evidence supports the notion that FGFs may mediate the predominantly osteoblastic phenotype of metastasis in patients with prostate cancer, like TGFβ, there are presently no direct associations between tumor-produced FGFs and osteoblastic metastasis.

v. BMPs. BMPs are bone-derived polypeptides and, with the exception of BMP-1, are members of the extended TGFβ superfamily. At least 15 members are currently recognized, and the list is growing. BMP-2 through BMP-8 share some TGFβ-related gene sequences. BMPs are synthesized by bone cells locally and stimulate the formation of ectopic bone when injected intraperitoneally or subcutaneously into rodents (418). BMPs stimulate the replication and differentiation of normal cells of the osteoblast lineage and, in contrast to TGFβ, enhance the expression of the differentiated osteoblastic phenotype (378, 419). BMP-1, -2, -3, -4, and -6 are temporally expressed in primary cultures of fetal rat calvarial cells (420). BMP-2, -4, and -7 have been shown to induce differentiation of primitive mesenchymal cells into bone when implanted into subcutaneous tissue (421). BMP-2 accelerates differentiation in primary cultures of fetal rat calvarial cells as demonstrated by an increase in expression of alkaline phosphatase and osteocalcin (421). BMP-3 decreases osteoclastic bone resorption and is chemotactic for monocytes. BMP-7 (osteogenic protein-1) suppresses cell proliferation and stimulates the expression of markers characteristic of the osteoblast phenotype in rat osteosarcoma cells but stimulates growth and differentiation in rat calvarial cultures (422). *In vivo,* human BMP-7 was capable of inducing new bone formation in the rat subcutaneous bone induction model (423). Recently, overexpression of BMP-4 in lymphocytes was described in association with the disabling ectopic osteogenesis of fibrodysplasia ossificans progressiva (424).

Normal and neoplastic prostate tissue express BMP-2, -3, -4, and -6 mRNA. The predominant form in normal human prostate tissue was shown to be BMP-4. While this pattern was observed in human prostate cancer cell lines, PC-3 and DU-145, PC-3 also expressed BMP-2 and -3 in large amounts. The rat prostate cancer PAIII expressed predominantly BMP-3 mRNA (425). PAIII is a cell line derived from a strain of rats, Lobund-Wistar, that has a 10% frequency of spontaneous prostate adenocarcinoma (426). PAIII stimulates new bone formation in this strain of rats, as well as in nude mice, when inoculated over the scapula. Rat BMP-3 was isolated from PAIII cells (427), and transfection of the PAIII tumor cells with a BMP-3 antisense construct somewhat reduced the osteoblastic response (428). Thus, biologically active BMPs expressed by prostate tumor in bone may contribute to the new bone formation at metastatic tumor sites in bone.

vi. Endothelin-1 (ET-1). ET-1 is the most recent factor implicated in the genesis of osteoblastic metastases. It is a potent vasoconstrictor and was originally purified from endothelial cells (429). Prostatic epithelium produces ET-1, and high-affinity ET-1 receptors are present throughout the prostate gland (430). ET-1 concentrations in seminal fluid are 500 times greater than those in plasma. ET-1 stimulates mitogenesis in osteoblasts, and osteoblasts have high-affinity receptors for ET-1 (431, 432). Osteoclastic bone resorption and osteoclast motility are decreased by ET-1 as well (433). Moreover, mean plasma endothelin concentrations in men with advanced, hormone-refractory prostate cancer with bone metastases were significantly higher than those concentrations in men with organ-confined prostate cancer or normal controls (417). However, these endothelin measurements were not correlated to tumor burden in bone and did not correlate with serum PSA concentrations. Human prostate cancer cell lines, DU-145, LNCaP, PC3, PPC-1, and TSU, have been shown to express ET-1 by RT-PCR. Finally, *in vivo,* ET-1 stimulated BMP-induced bone formation as assessed by alkaline phosphatase activity in a rat model of matrix-induced bone formation (434). Additionally, IL-6, but not estrogen, tamoxifen, TGFβ, TNF, γ-interferon, or IL-1, stimulated ET-1 production from human breast cancer cells MCF-7 and ZR-75–1 (435). This is of interest since breast cancer is occasionally associated with osteoblastic metastasis.

Thus, the mechanisms responsible for the predominantly osteoblastic phenotype of prostate cancer metastatic to bone is complex and likely is the result of multiple tumor-produced factors on normal bone remodeling. Figure 8 is a schematic model based on available data from the literature that identify potential tumor-bone interactions.

c. Bone microenvironment as the soil for prostate cancer: As bone matrix is an abundant source of growth factors, some of which are released as a consequence of osteoclastic bone resorption, it is likely a fertile soil for prostate cancer cells as well as breast cancer cells. For example, human prostate cancer cell lines proliferate in response to conditioned media from human, rat, or bovine bone marrow (436). Conditioned media from osteoblast-like cells enhance growth of LNCaP, PC-3, and DU-145 (437). Other in vitro studies indicate that TGFβ stimulated adhesion of the human prostate cancer cell line PC3 to bone matrix and that this adhesion appears to be mediated via $\alpha_2\beta_1$ integrins (438). TGFβ has also been shown to stimulate cell motility of the MATLyLu, an *in vitro* observation that suggests that bone-derived TGFβ may be an important chemotactic factor in prostate cancer (439). EGF, secreted by MG-63 bone cells stimulated chemomigration of the TSU-pr1 prostate cancer line in Boyden chambers (440).

3. Hematogenous. Multiple myeloma is a plasma cell malignancy that is almost invariably associated with destructive bone lesions, either in the form of diffuse osteopenia or localized osteolysis throughout the skeleton (441). Eighty percent of patients with myeloma first present with pain, which can be related to the bone disease, and bone complications are the most obvious clinical feature in most patients

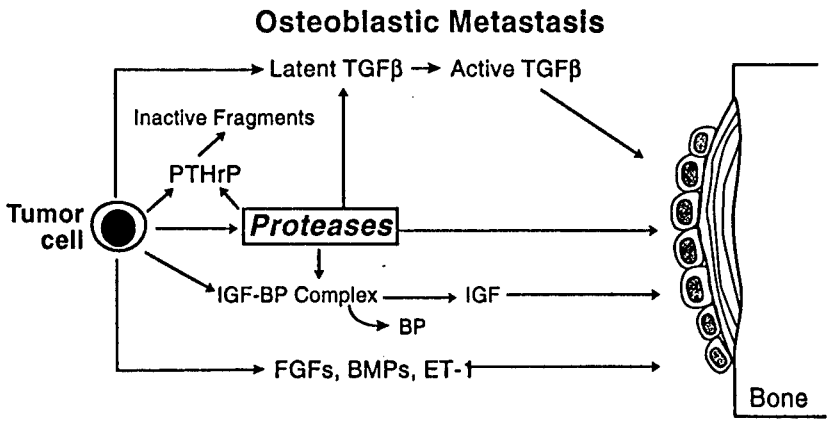

FIG. 8. Model for the formation of osteoblastic bone metastases from prostate cancer. Tumor production of factors such as TGFβ, FGFs, BMPs, and ET-1 may directly stimulate osteoblastic activity and subsequent bone formation. Proteases such as PSA, uPA, and cathepsin D may activate latent TGFβ, release IGFs from inhibitory binding proteins, and inactivate PTHrP.

(441). The bone lesions of myeloma may be diffuse or localized and comprise three types. In the majority of patients (more than 95%), they are osteolytic. These lesions occur predominantly in those bones that are rich in red marrow, *e.g.*, the axial skeleton, and are associated with increased osteoclast activity adjacent to sites of myeloma cell accumulation. This suggests that myeloma cells produce locally active soluble factors that stimulate the remaining osteoclasts to resorb bone (442). In some patients, the bone loss is more generalized and its appearance more closely resembles that of osteoporosis. In these patients the myeloma cells tend to be more diffusely spread throughout the axial skeleton. Some patients may have a combination of these two pictures, *i.e.*, osteopenia of vertebral bodies but discrete osteolytic bone lesions of the skull. Myeloma bone disease is always an important differential diagnosis in the patient who presents with apparent osteoporosis. The bone disease of myeloma tends to be steadily progressive in most patients and can be used as one of the parameters to monitor the course of the disease. For example, obvious progression of the bone lesions or the appearance of new discrete lesions indicates that the disease is active. On the other hand, vertebral body collapse can occur in patients in remission because of the weakened state of the skeleton, and bone pain itself is not a reliable indicator of the state of the disease activity.

The type of osteolytic disease that occurs in myeloma may be quite different from the bone disease associated with other types of malignancy, such as carcinoma of the breast. In myeloma bone disease there is often no increase in bone formation or osteoblast activity. The reason for this complete uncoupling of bone formation and bone resorption is unknown. It is paralleled clinically by the absence of an increase in the markers of bone formation, which are frequently present in other types of osteolytic bone disease due to malignancy, such as serum osteocalcin and alkaline phosphatase (161). In addition, the radionuclide bone scan shows no evidence of increased isotope uptake at the site of bone lesions.

A small number of patients with myeloma bone disease present with an entirely different picture of diffuse osteosclerosis (279, 443). Osteosclerotic myeloma often occurs as a part of a syndrome of polyneuropathy and is associated with the cutaneous and endocrine features that comprise POEM's syndrome (polyneuropathy, organomegaly, endocrinopathy, M-protein, and skin changes) (444).

Thus, the majority of patients with myeloma have a crippling form of bone disease associated with intractable pain, susceptibility to fracture upon trivial injury, and nerve compression syndromes (most frequently spinal cord compression), associated with vertebral body collapse. About 30% of patients develop hypercalcemia at some stage during the course of the disease, usually in association with impaired renal function (441). The pathophysiology of bone destruction that is so characteristic of myeloma bone disease remains unclear. Cultures of myeloma cells *in vitro* produce several osteoclast activating factors such as IL-6, IL-1β, and TNFβ or lymphotoxin (445–447), which have been implicated. Lymphotoxin (or TNFβ) is a powerful bone-resorbing cytokine that is produced by lymphoid cell lines *in vivo*. It is expressed by many B cell lines in culture, including cell lines derived from patients with myeloma (447). It is capable of causing hypercalcemia *in vivo* and has effects on bone resorption that are indistinguishable from those of TNFα, namely an increase in osteoclast formation and activity, associated with impaired osteoblast differentiation and increased osteoblast precursor proliferation (224). Although produced by many cell lines derived from patients with myeloma, it has not been detected in freshly isolated marrow cells from these patients.

The second cytokine that has been implicated in the bone lesions in myeloma is IL-1β (445, 446). IL-1β is also a powerful stimulator of osteoclastic bone resorption, which increases osteoclast formation and osteoblast proliferation and can cause hypercalcemia *in vivo*. Freshly isolated marrow cells from patients with myeloma, which contain myeloma cells plus other marrow cells, have been shown to contain IL-1β in the conditioned medium harvested from these cells. Moreover, bone-resorbing activity present in these conditioned media can be neutralized by antibodies to IL-1β.

Finally, IL-6 is a cytokine that has an important growth-regulatory role in many patients with myeloma (448). IL-6 concentrations in bone marrow and in plasma correlate with the stage of disease (449–451). It may be produced by the myeloma cells or other stromal cells in the marrow microenvironment. Endogenous IL-6 production is 2–30 times greater in patients with myeloma than in normals (450). Administration of a murine-human chimeric anti-IL-6 antibody to patients with multiple myeloma resistant to second-line chemotherapy suppressed this IL-6 production, but did not prevent infection-induced IL-6 production (450). Such

results suggest that this IL-6 antibody inhibited a positive feedback IL-6-dependent loop.

IL-6 is not a powerful bone-resorbing factor in its own right, but is capable of enhancing the effects of other factors on bone resorption, presumably by increasing generation of precursors for cells in the osteoclast lineage (165). Myeloma cells occasionally produce PTHrP and since IL-6 may potentiate the osteoclastic bone resorption mediated by PTHrP, such cytokine interactions have important implications in the genesis of the bone destruction associated with myeloma.

Just as there may be a vicious cycle in the bone microenvironment between the tumor cells and bone-derived growth factors in breast cancer, there may be a similar relationship between the process of bone resorption and myeloma cell behavior (Fig. 9). In this latter case, the responsible mediator may be IL-6, which is the major growth factor for myeloma cells. In myeloma, stimulation of bone resorption may lead to IL-6 generation (452), which in turn may be responsible for maintaining aggressive growth of the malignant cells. Primary tumor cells from myeloma patients induced IL-6 secretion by adherent cells from long-term bone marrow cultures (451). Interestingly, this IL-6 production did not occur when tissue-culture inserts, which prevented direct contact between the adherent cells from bone marrow and the myeloma cells, were used in the wells. Such findings imply an important role for cell-cell contact in mediating the bone marrow cell induction of IL-6 by myeloma cells. Binding of myeloma cells to adherent cells from long-term bone marrow cultures was partly inhibited by antibodies against the adhesion molecules, very late antigen-4 (VLA-4), CD44, and lymphocyte function-associated antigen 1 (LFA-1), as was IL-6 production (451). These data indicate that one source of IL-6 may be the normal cells in the bone marrow and that adhesion molecules expressed on myeloma cells may mediate the induction of IL-6.

Other properties of myeloma cells contribute to its predilection to grow in the bone microenvironment. Cell surface adhesion molecules expressed by myeloma cells may bind to bone marrow stromal or endothelial cells. A variety of adhesion molecules, such as the VLAs, intercellular adhesion molecules, and LFAs (453–458), are expressed on myeloma cells. VLA-5 and MPC-1 were reported to be involved in the adhesion of mature myeloma cells to bone marrow stromal cells (459). Additionally, expression of the cell surface adhesion molecule CD21 was demonstrated on both mature and immature myeloma cells. CD23 present on bone marrow stromal cells bind CD21 and, thus, may mediate myeloma cell adhesion in the bone microenvironment (460). VLA-4 is the principal integrin present on the U266, ARH-77, IM-9, and HS-Sultan human myeloma cell lines, and work by Uchiyama *et al.* (454) reveals that such cells bind fibronectin through VLA-4 as well as through RGD-dependent mechanisms. This binding was down-regulated by IL-6. Studies from human subjects with myeloma and monoclonal gammopathy of unknown significance indicate that expression of plasma cell adhesion molecules LFA-1, VLA-4, and CD44 was highest in myeloma patients and was associated with increased evidence of angiogenesis (461). Finally, CD44 expression by myeloma cells may mediate colonization in bone by binding osteopontin (311).

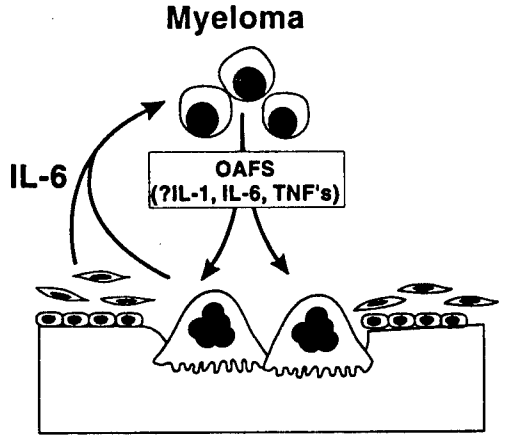

FIG. 9. Model for the bone destruction associated with myeloma. Myeloma cells produce osteoclast activating factors (OAFs) which stimulate osteoclastic bone resorption. IL-6 derived from the osteoblast, osteoclast, stromal cell, and myeloma cell itself stimulate growth of the myeloma cells.

At this point in time, it is not possible to say which is the most important cytokine involved in myeloma. The bone disease may be due to the combination and interaction of a number of cytokines and other molecules working in parallel, derived from both myeloma as well as from the normal host cells. Several animal models of myeloma bone disease have been developed recently that should provide significant insight into the pathophysiology of this disease (462, 463).

Until recently, the management of myeloma bone disease comprised treatment of the underlying malignancy, management of bone pain with analgesics, radiation therapy if the bone disease was localized, and cautious treatment of hypercalcemia when it occurred because it is likely accompanied by impaired glomerular filtration. Recently, this has changed with the availability of more potent bisphosphonates. The Food and Drug Administration approved the use of pamidronate for myeloma bone disease in 1995. Pamidronate has been shown to reduce skeletal events associated with myeloma, including the need for radiation therapy and pathological fractures. It also probably reduces the number of episodes of hypercalcemia. When given in doses of 90 mg by intravenous infusion over 4 h monthly for 9 months, it has been shown to be very effective in patients with advanced disease, and the number of skeletal events is almost halved in patients taking pamidronate. Moreover, an objective assessment of quality of life in treated patients has shown a beneficial effect of pamidronate (464). Similar studies in Europe have also suggested that potent bisphosphonates such as clodronate are effective in improving performance status, reducing bone pain, vertebral fractures, and progression of osteolytic bone lesions, as well as preventing hypercalcemic episodes. Animal studies using a mouse model of myeloma with the bisphosphonate, risedronate, reveal similar findings (465). Previous clinical studies with less potent bisphosphonates in patients with myeloma have been less successful as oral etidronate was ineffective (466) and oral clodronate inhibited the progression of osteolytic bone lesions but did not reduce bone pain or fracture rate (467). Clinical studies in patients with myeloma indicate that adjuvant treatment with

low-dose gallium nitrate attenuates the rate of bone loss as well as the associated bone pain (468). There is no definitive evidence as yet for a beneficial effect of inhibition of bone resorption on tumor burden. Bisphosphonates seem to be most effective in patients with minimal disease, but are appropriate for almost all patients.

As with solid tumors that cause destructive bone lesions and hypercalcemia, hypercalcemia is also frequent in myeloma, occurring in about 30% of all patients sometime during the course of the disease. Hypercalcemia is always associated with bone destruction and often with impaired renal function. When hypercalcemia occurs in patients with myeloma, it is usually accompanied by impaired renal function, which limits the availability of specific therapies for treatment of the hypercalcemia. Under these circumstances, the most appropriate medical agent to use is a new generation bisphosphonate, although experience with these drugs is limited in patients with markedly impaired glomerular filtration. An alternative is the use of a combination of calcitonin and glucocorticoids, which has been shown to be very effective in reducing hypercalcemia in patients with myeloma (67, 248). However, since calcitonin must be given by injection, this combination is not as convenient as the new generation bisphosphonates.

In myeloma, bone destruction occurs as a consequence of osteoclastic bone resorption, as osteoclasts accumulate on bone-resorbing surfaces adjacent to myeloma cells. In an early clinical study of patients with myeloma, biopsy samples indicate that active osteoclastic bone resorption correlated with the presence of greater than 20% myeloma cells in the adjacent marrow cell population (442, 469). The striking feature in myeloma is that there is a marked increase in osteoclastic bone resorption, usually without manifestations of increased bone formation (469). These abnormalities in bone formation were confirmed by transiliac bone biopsies from 118 patients with myeloma in which quantitative bone histomorphometry demonstrated that osteoid seams were reduced in thickness and had a lowered calcification rate (470). This is in contrast to breast cancer, where although the bone lesions are mainly destructive, there is usually a slight increase in bone formation and an increase in serum alkaline phosphatase and radionuclide uptake at sites of osteolytic deposits associated with increased osteoblast activity. The mechanism of this uncoupling of bone formation from bone resorption is not known but is the subject of intense study.

D. Therapy of tumor in bone

Most patients with bone metastasis are normocalcemic. In a majority of breast cancer patients with bone metastases, local osteolysis occurs without hypercalcemia (268), increases in nephrogenous cAMP (69), or increases in PTHrP (111). Osteolytic bone lesions are most frequent in patients with carcinoma of the breast, carcinoma of the lung, and myeloma, the same malignancies that are associated with hypercalcemia. However, there are also other solid tumors in which hypercalcemia is rare but osteolytic bone lesions are relatively frequent. These include patients with carcinoma of the thyroid. These patients suffer considerably because of their bone lesions, which cause intractable pain, pathological fracture after trivial injury, nerve compression syndrome such as spinal cord compression, and propensity to develop hypercalcemia. Until recently, therapy of tumor in bone was directed against tumor cells for breast and prostate cancer as well as myeloma and other malignancies. This usually involved chemo- or hormonal therapy, local field irradiation, radionuclide therapy, or surgery (274, 471, 472). The advent of bisphosphonates has changed this perspective somewhat in that it has added therapy directed against the osteoclast to our current armamentarium of anticancer drugs.

As metastatic bone disease is mediated by osteoclastic bone resorption, and factors that stimulate osteoclastic bone resorption, such as PTHrP, enhance bone destruction by tumor, it is logical to consider therapy with inhibitors of bone resorption to prevent the development of bone metastasis or to delay their progression. Other mechanisms by which bisphosphonates exert their effects to decrease bone metastases may involve tumor cell adhesion to bone. *In vitro* studies demonstrate that a number of bisphosphonates decrease attachment of MDA-MB-231 breast cancer cells to extracellular bone matrix. Of interest is the fact that the effect of these bisphosphonates to decrease tumor adhesion positively correlated to the antiresorptive potency (473).

Several prospective, double-blind placebo-controlled trials have been published documenting the efficacy of the bisphosphonate, pamidronate, in decreasing the skeletal complications associated with breast cancer (321) and myeloma (464). For patients with myeloma, the Food and Drug Administration (FDA) has recently approved pamidronate for use in patients with osteolytic lesions who are not hypercalcemic. This is based on a recent study (464), which shows that intravenous pamidronate given every 4 weeks for nine cycles in almost 400 patients with myeloma caused a significant reduction in skeletal complications (defined as pathological fracture, requirement for radiation to bone or surgery, or spinal cord compression), decreased the occurrence of new pathological fractures, and prevented development of hypercalcemia. In addition, this treatment alleviated bone pain and improved quality of life. There was a suggestion in these patients that there may have also been a beneficial effect on overall survival. Pamidronate is therefore now being widely used early in the course of myeloma since it is a relatively nontoxic drug and may have a beneficial effect not only on bone complications. There is no definitive evidence as yet for a beneficial effect on tumor burden or survival, and this will require careful controlled studies in more extended numbers of patients.

Clinical studies have been ongoing for 20 yr in normocalcemic patients with solid tumors and osteolytic bone metastases. All of the available evidence from these studies suggests that drugs that decrease bone resorption, such as the potent bisphosphonates, have a beneficial effect on skeletal complications, including pain and pathological fracture, prevention of hypercalcemia, and improved quality of life. In a recent multicenter trial which consisted of more than 700 patients with stage IV breast cancer with two or more predominantly lytic lesions, with at least one lesion that was 1 cm or greater in diameter, treatment with pamidronate 90 mg iv every 3 to 4 weeks for 12 months in conjunction with chemotherapy or hormonal therapy resulted in a significant

reduction in skeletal complications and bone pain compared with the control group (321). However, there may be an added beneficial effect of the bisphosphonates that is even more important. In experimental studies in which human breast cancer cells are inoculated into the left ventricle of the nude mouse, Sasaki *et al.* (296) have shown that bisphosphonates such as risedronate and ibandronate not only prevent the development of skeletal complications and bone metastases, but they also reduce tumor burden in bone. This likely occurs because the bisphosphonates make bone a less favorable environment for the growth of tumor cells by reducing bone turnover and decreasing the supply of local bone-derived growth factors that also act as tumor growth factors in the bone microenvironment. It is apparent from clinical studies that the use of bisphosphonates reduces significant skeletal morbidity in advanced breast cancer (312–323). These data suggest that drugs that inhibit bone resorption may be useful adjuvant therapy in patients with malignant disease by preventing the growth of tumor cells in the skeleton.

Studies using bisphosphonates in the treatment of prostate cancer metastatic to bone are fewer and less impressive. Clodronate treatment of men with advanced prostate cancer not only resulted in decreased osteoclastic bone resorption, as assessed histomorphometrically, but also in osteomalacia. The authors explained the transient relief of bone pain in the clodronate group to this resultant osteomalacia (474). In a small study of breast and prostate cancer patients with osteosclerotic lesions, treatment with pamidronate resulted in a decrease in bone pain. This response was mostly predicted by a decrease in the urinary marker of bone resorption, deoxypyridinoline (475).

Despite the encouraging results presented in these and other studies, several important questions remain regarding the use of bisphosphonates for treatment of tumor in bone. 1) Will bisphosphonates be useful as adjuvant therapy in tumor types other than breast cancer and myeloma? 2) Will bisphosphonate treatment in cancer improve survival? 3) Will bisphosphonates be beneficial in prevention of bone metastasis if therapy is initiated before the development of bone metastasis in patients with limited disease? 4) Will bisphosphonate therapy in cancer prove to be cost effective? 5) Is there a role for bisphosphonate therapy in osteoblastic metastasis? Although animal studies suggest that the answers to these questions may already be obvious, only prospective trials in humans will provide us with the definitive answers.

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Endocrine-Related Cancer (1999) **6** 1-99

Factors regulating the growth of metastatic cancer in bone

B F Boyce, Toshiyuki Yoneda[1] and T A Guise[1]

Department of Pathology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78284, USA

[1]Department of Medicine, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78284, USA

(Requests for offprints should be addressed to B F Boyce)

Abstract

Metastatic tumor cells can interfere directly with the function of bone cells involved in normal bone remodeling or indirectly by influencing the behavior of hematopoietic, stromal and other cells in bone marrow that interact with bone cells. Recent studies of metastatic cancer have revealed that tumor cells interact closely with vascular endothelial cells, basement membrane and bone marrow stromal cells through cell surface proteins or by releasing factors which affect the function of these cells. Bidirectional interaction between marrow cells and tumor cells can give the latter a selective advantage for growth in bone which can lead to the destruction of or to increased production of bone matrix. Understanding of the mechanisms involved in tumor metastasis and growth in bone has increased in recent years, and in this review we shall describe current knowledge of these mechanisms and of the predilection of certain types of cancers to metastasize to bone, their growth in the bone microenvironment and interactions between them and bone cells. Because metastatic breast cancer has been studied more than any other, we shall focus on it as a representative example, although the general principles apply to other types of cancer and to myeloma.

Endocrine-Related Cancer (1999) **6** 1-99

Bone modeling and remodeling

A firm grasp of the sequence of cellular events involved in the process of bone modeling and remodeling is essential for understanding the effects of tumor cells on the skeleton, but it is outside the scope of this review to describe these in detail here. However, because we shall be describing a number of models in which growing animals are used to examine the effects of tumors on the skeleton, bone modeling and aspects of bone remodeling relevant to the effects of malignancy will be covered briefly.

Long bones are sculpted from a cartilage analage formed in limb buds during embryonic development and increase in length through endochondral ossification in which cartilage is calcified and subsequently replaced by bone matrix near the ends of the bones (reviewed by Panganiban *et al.* 1997, Shubin *et al.* 1997). They increase in width by periosteal matrix apposition by osteoblasts, and the marrow space is increased in diameter in proportion to the length of the growing bone by endosteal resorption of cortical bone by osteoclasts. New cancellous bone is laid down rapidly following resorption of calcified cartilage at the growth plate, but most of it is quickly removed by osteoclastic resorption to maintain a medullary cavity as the bone grows. Mutations in genes regulating limb bud development lead to dwarfism or reduced numbers of skeletal elements, while defects in osteoclast generation or function result in build up of cancellous bone within the medullary cavity - the hallmark of osteopetrosis (reviewed by Mundlos & Olsen 1997, Boyce *et al.* 1999). Metastases to long bones of growing rodents used in experimental studies of the effects of tumors on the skeleton can interfere with normal bone modeling and endochondral ossification, and intervention therapy, such as bisphosphonates, given to inhibit bone resorption in such studies can result in a build up of unresorbed bone, similar to that seen in osteopetrosis (Sasaki *et al.* 1995).

Cancellous (also called trabecular) bone remains at the ends of long bones in humans after epiphyseal closure and, like cancellous bone in the axial skeleton, is remodeled throughout life. Bone remodeling begins on bone surfaces with a team of osteoclasts resorbing a trench of bone approximately 60 μm deep. Osteoblasts then lay down

Table 1 Agents released by tumor cells that stimulate bone resorption

Colony stimulating factors (CSFs)
Epidermal growth factor (EGF)
Interleukin-1 (IL-1)
Oxygen-derived free radicals
Platelet-derived growth factor (PDGF)
Prostaglandins
Parathyroid hormone (PTH)
Parathyroid hormone-related protein (PTH-rP)
Tumor necrosis factor α (TNF α)
Tumor necrosis factor β (TNF β; lymphotoxin)
Transforming growth factors α and β (TGF α, TGF β)

new bone matrix in the trench in a process with many similarities to the repair of damaged sections of roadways. The two processes of resorption and formation are coupled temporally in this site-specific manner and it is estimated that there are about one million bone remodeling units in the normal adult skeleton at any time. The primary function of bone remodeling is thought to be the removal of worn-out sections of bone that have become weakened with age. The number of bone remodeling units is increased in conditions such as estrogen deficiency, hyperparathyroidism and Paget's disease, in which bone turnover is increased.

Osteoclasts are derived from precursors in the mononuclear-phagocyte lineage under the combined influence of macrophage colony-stimulating factor and the recently identified osteoclastogenic cytokine, known as RANK ligand, OPG ligand, TRANCE, and ODF (Anderson *et al.* 1997, Wong *et al.* 1997, Lacey *et al.* 1998, Yasuda *et al.* 1998). Osteoblasts are derived from the stromal cell lineage and in addition to laying down type I collagen, the major structural protein in bone, they secrete a number of growth factors into bone matrix (Hauschka *et al.* 1986). Hematopoietic tissue and stromal cell precursors persist at the ends of long bones and within the medullary cavities of vertebral bodies and co-operate with bone cells in regulating remodeling in these bones. As will be seen later, bone remodeling can significantly influence the function of metastatic tumor cells because the intense resorption of new bone matrix near growth plates and the resorption of remodeling bone elsewhere are associated with the release of growth factors from bone into the local microenvironment and these can promote tumor cell proliferation and osteolysis preferentially at these sites. In contrast, the diaphyseal cavity of long bones in humans is normally devoid of trabecular bone and is filled with fatty marrow. Metastatic tumor deposits are found much less frequently at these sites than in the axial skeleton or the ends of long bones, presumably because there is less bone remodeling on the diaphyseal endosteal surfaces than at these other sites. Although these differneces in bone volume and thus in bone remodeling rates between diaphyseal lung bones and cancellous bone sites may account for the increased occurrence of metastases in the latter, they do not ecplain why metastases may occur in particluar vertebrae, for example, while sparing others. This is likely to be the result of the interplay among a variety of factors, including blood flow rates, relative vascularity and bone remodelling rates at particular sites in the skeleton at the time tumor cells are circulating in the blood. However, we are uunaware of any studies that have examined this aspect of bone metastasis sepecifically.

Local effects of tumor cells on bone

Some malignant tumours, such as breast, lung and prostate, have a predeliction to spread to bone and typically cause osteolytic (breast and lung), osteoblastic (prostate) or mixed osteolytic and osteoblastic metastases (Mundy & Martin 1993). In addition to these solid tumors, myeloma typically causes extensive bone destruction and hypercalcemia (Mundy 1995). In all of these circumstances, the bone is resorbed by osteoclasts stimulated by tumor cell products, rather than by the tumor cells (Francini *et al.* 1993, Taube *et al.* 1994, Yoneda *et al.* 1994, Mundy & Yoneda 1995). Malignant tumors can also cause humorally mediated hypercalcemia by releasing factors, such as parathyroid hormone-related hormone (PTHrP), that act systemically to increase bone resorption and enhance renal tubular reabsorption of calcium. This subject has been reviewed elsewhere recently (Guise & Mundy 1998) will not be covered here.

Bone metastases can cause bone pain, pathologic fractures, nerve compression syndromes (especially in myeloma) and hypercalcemia mainly as a result of the effects of growth factors, cytokines and hormones released into the bone marrow around them (Table 1). These act in a number of ways to increase osteoclast numbers and activity. For example, interleukin (IL)-6 which is released by myeloma cells, promotes the proliferation of early osteoclast precursors. However, it does not enhance fusion of the precurors to form osteoclasts and has only weak stimulatory effects on osteoclasts. In contrast, IL-1 which is produced by some solid tumors (Sato *et al.* 1988), stimulates osteoclast formation and activity (Uy *et al.* 1995), in part by prolonging their life spans (Jimi *et al.* 1998) through prevention of apoptosis (Hughes *et al.* 1994). PTHrP which is released by many tumor cell types (Danks *et al.* 1989, Asa *et al.* 1990, Dunne *et al.* 1993) has effects on osteoclasts similar to those of IL-1 although, unlike IL-1, it does not stimulate the

production of granulocyte-macrophage colony-forming units from which osteoclast precursors arise (De La Mata *et al.* 1995). Release of PTHrP is often associated with hypercalcemia because it also enhances renal tubular reabsorption of calcium.

Predilection for breast cancer to metastasize to bone

The predilection for breast cancer to metastasize to bone was described more than 50 years ago by Walther (1948). He found in an autopsy study (in which the confounding influence of chemotherapy was not a factor) that 64% of 186 patients who died of breast cancer had metastases to bone. Two more recent studies reported that 71% of 707 (Cifuentes & Pickren 1979) and 62% of 1060 (Weiss 1992) breast cancer patients had bone metastases at autopsy, suggesting that chemotherapy has not affected the predilection for or frequency of metastasis to bone. Furthermore, in another study in which 69% of 587 patients dying of breast cancer had bone metastases (Coleman & Rubens 1987), bone was the most common site of first distant relapse. Thus, bone metastasis is a common complication of breast cancer and one for which only palliative therapy is presently available.

Two major factors determine the dissemination of cancers to distant organs: the biological properties of the cancer cells and the environment at the metastatic site. Metastasis to and growth of tumor cells in distant organs involve multiple and complex steps (Fidler 1990, Liotta 1992). Cancer cells with a high predilection for metastasis to bone must have properties not present in tumors that rarely spread to bone. These could include production of proteolytic enzymes, angiogenic factors, autocrine growth-stimulating factors, increased expression of growth factor receptors, temporal and spatial expression of cell adhesion molecules (CAMs) and resistance to host immune surveillance. However, most metastatic cancers are likely to possess many of these properties, and thus other characteristics must account for the preferential colonization of bone by breast cancer.

Metastatic cancer cells enter bone mainly through nutrient arteries and these communicate with a sinusoidal network in the bone marrow (DeBruyn 1981, Yoneda 1997) rather than with a capillary system found in most solid organs. They express CAMs to establish contact with sinus endothelial cells and secrete proteolytic enzymes to degrade the endothelial wall and pass into the bone marrow compartment. Certain cancer cells attach preferentially to endothelial cells in particular organs (Auerbach *et al.* 1987), suggesting a contributory role of endothelial cells in organ preference. Furthermore, protease secretion by cancer cells is also influenced by target organs (Nakajima *et al.* 1990). The particular cell-cell adhesion molecules and proteases involved in breast cancer cell attachment and invasion in bone are as yet unidentified.

There are numerous types of cells in bone marrow involved in the maintenance of immune and inflammatory responses and in blood cell homeostasis. The activities of these cells which include lymphocytes, plasma cells, macrophages and erythroid and myeloid precursors are regulated by a variety of cytokines and growth factors and complex cytokine growth factor networks. Breast cancer cells migrating into the bone marrow are exposed to cells producing these cytokines and growth factors, and it is likely that they interact with one another to enhance tumor cell growth and activity. The progression of osteolytic metastases requires the establishment of functional interactions between metastatic breast cancer cells and osteoclasts. These interactions could be mediated by direct cell-cell contact and/or production of soluble stimulators of osteoclast activity (Yoneda *et al.* 1994, Mundy & Yoneda 1995, Yoneda 1998).

Several genes have been identified recently that may contribute to the metastatic potential of breast cancer. An anti-metastatic gene (NM23) was originally cloned from low metastatic murine melanoma cells using subtractive hybridization techniques (Steeg *et al.* 1988) and high expression of it is associated with a good prognosis in breast cancer patients (Hennessy *et al.* 1991, Hirayama *et al.* 1991). Decreased expression of a metastasis suppressor gene called KA11 has been observed in metastatic human prostate cancer cells (Dong *et al.* 1995), while a metastasis-promoting gene named MTA1 has been cloned from rat mammary adenocarcinoma cells using differential hybridization (Toh *et al.* 1994). Since the protein products of these genes have not yet been characterized, it is not known if they play a role in breast cancer metastasis to and organ selectivity for bone.

The bone microenvironment

The observation that most cancers exhibit target organ preference when they disseminate was first reported by Paget (1889) in a study of autopsy records of 735 women who died of breast cancer. He found that the highest numbers of metastases were in the ovaries, followed by the skeleton. On the basis of these observations, he proposed the 'Seed and Soil' theory that the microenvironment of the organs to which cancer cells spread serves as a fertile soil for their growth. This hypothesis has been widely accepted as a basic principle in the field of cancer metastasis (for review see Rusciano & Burger 1992), and we believe it is particularly relevant to bone.

During bone formation, osteoblasts lay down a variety of growth factors which become incorporated into bone matrix along with type I collagen (Hauschka *et al.* 1986). These are released in active form into the marrow when

bone matrix is degraded during osteoclastic resorption (Pfeilschifter & Mundy 1987). Many of these growth factors, which include tranforming-growth factor (TGF) β, fibroblast growth factors (FGF), insulin-like growth factors (IGF) and bone morphogenetic proteins (BMP) could stimulate the growth of metastatic cancer cells in the marrow. Among these, IGFs, whose concentrations in bone are higher than those of other growth factors (Hauschka *et al.* 1986), promote the growth of human breast cancer MDA-MB-231 cells (Yoneda *et al.* 1995). Furthermore, the mitogenic activity of culture supernatants from resorbing bone on MDA-MB-231 cells was decreased by the addition of neutralizing antibodies to the IGF-I receptor (Yoneda *et al.* 1995). TFGß has been shown to stimulate the proliferation of Walker 256 carcinosarcoma cells that metastasize to bone (Orr *et al.* 1995).

Further evidence that the target organ host environment influences the metastatic potential of cancers is the observation that some cancers increase their metastatic and organ-preferential properties by successive *in vivo* passages through target organs. For example, murine melanoma B16 cells with low metastatic potential become highly metastatic to lung (B16F10) and liver (B16L8) after repeated selection and culture from pulmonary (Hart & Fidler 1981) or hepatic (Tao *et al.* 1979) metastatic foci respectively. B16L8 cells respond specifically to growth factors from hepatocytes, whereas B16F10 do not (Sargent *et al.* 1988). Other examples include human colon cancer cells which developed high metastatic ability and organ selectivity for the liver (Morikawa *et al.* 1988) following repeated passage, and this was associated with expression of greater numbers of functional receptors for TGFα and hepatocyte growth factor than colon cancer cells with low metastatic potential (Fidler 1995). Although it is unclear from these studies whether the multiple *in vivo* passages resulted in enrichment of highly metastatic and organ-preferential subpopulations of cancer cells or in acquisition of metastatic ability and organ preference, they demonstrate that the metastatic potential and behavior of cancer cells can be altered by specific organ environments.

Growth factors present in bone could also contribute to the bone preference of breast cancer by being chemotactic for tumor cells. For example, culture supernatants of resorbing bone (Orr *et al.* 1979) and type I collagen and its fragments (Mundy *et al.* 1981) released during bone resorption stimulate chemotaxis of breast cancer cells in a Boyden chamber assay. Thus, following entry to the bone marrow space, breast cancer cells might migrate preferentially to resorbing bone surfaces in response to an increasing local concentration gradient of bone products, such as type I collagen, and then be exposed to high local levels of bone-derived growth factors that could promote their proliferation.

Although bone provides a favorable environment for proliferation of metastatic breast and other cancer cells, this alone cannot account for the special prospensity of these cells to thrive in it, since other metastatic cancer cells passing through bone are likely to be exposed to the same influences. For example, as outlined in detail later, the release of factors such as PTHrP by breast cancer cells could stimulate osteoclastic resorption around them resulting in the release of more growth factors from bone, thus establishing an up-regulatory cycle between these cell types.

Experimental approaches to study breast cancer metastasis to bone

Detailed study of the metastatic behavior of cancers and of the complex interactions between cancer cells and host cells requires the development of good animal models. The ideal model would be one in which cancer develops spontaneously in the organ of interest and metastasizes to bone. However, these models are extremely difficult to establish. Thus, few are available (Orr *et al.* 1995) and there are no spontaneous models of breast cancer metastatic to bone.

This problem can be partly circumvented in models in which cancer cells are injected directly into the blood stream. The cellular and molecular mechanisms of organ preference of metastatic tumors can be studied in such an experimental model because the steps involved before cancer cells reach their preferential target organs are likely to be non-specific and independent of organ selectivity. To this end, we modified the model originally described by Arguello *et al.* (1988) to develop an animal model of bone metastasis of human breast cancer in nude mice (Yoneda *et al.* 1994). We injected the human breast cancer cell line, MDA-MB-231, into the left cardiac ventricle of female nude mice and found that these cells preferentially cause osteolytic bone metastases and rarely form metastases in other organs. More recently, we observed that these animals exhibit osteolytic metastases to the jaw, including mandible, maxilla, and zygomatic arch (Sasaki *et al.* 1998).

We monitored the development and growth of osteolytic lesions by serial radiography and quantitated the size of the lesions by computer-assisted image analysis. Animals rarely became hypercalcemic, but frequently developed cachexia and occasionally paraplegia, due to vertebral metastases. Replacement of the bone and marrow at the ends of long bones with metastatic breast cancer cells was confirmed histologically and numerous multinucleated osteoclasts were seen adjacent to cancer cells on the endosteal bone surface (Sasaki *et al.* 1995). Similar findings have been reported using the human

melanoma cell line, A375 (Nakai *et al.* 1992, Hiraga *et al.* 1995).

There are several other animal models of bone metastasis (Orr *et al.* 1995). These include the Walker 256 carcinosarcoma which metastasizes spontaneously to bone after intramuscular inoculation (Kostenuik *et al.* 1992) and the human PC3 prostate cancer which metastasizes to vertebrae in nude mice following tail vein injection if the vena cava is compressed beforehand to force the flow of blood and the injected tumor cells into the vertebrae via Barton's vertebral venous complex (Shevrin *et al.* 1988).

Because metastasis is a complex multistep process, certain aspects of it cannot be studied in detail using *in vivo* approaches and reproducible, quantitative and convenient *in vitro* assays are required. *In vitro* models for attachment to extracellular matrix, invasion, chemotactic migration and matrix metalloproteinase (MMP) production have been developed (Mareel *et al.* 1991*a*) for study of the general steps of cancer metastasis, but *in vitro* models for the study of metastasis to bone specifically are difficult. Currently, the effects of cancer products on bone resorption or osteoclast formation are generally studied using organ cultures of radiolabeled fetal rat long bones or mouse calvariae or bone marrow cells cultured in the presence of the culture supernatants of cancer cells. Culture supernatants of bone or osteoblasts can be assessed for their effects on cancer cell proliferation and production of proteases, cytokines and growth factors which promote osteoclastic bone resorption. However, additional *in vitro* assays which can examine other specific components of the *in vivo* processes of bone metastasis are needed to further advance the study of cancer metastasis to bone.

Growth of metastatic cancer and the bone microenvironment

One way to determine whether factors released during bone resorption promote the growth of metastatic cancer cells in bone is to inhibit bone resorption before or after tumor cells metastasize to bone. To test this hypothesis, we inoculated breast cancer cells into the left cardiac ventricle of nude mice and treated the mice with the bone resorption-inhibiting bisphosphonate, risedronate. We used three different protocols in which risedronate was given before, simultaneously with or after breast cancer cell inoculation. In these experiments, risedronate either prevented the development of new osteolytic bone metastases or decreased the progression of those already established. Importantly, histomorphometric analysis revealed that, in risedronate-treated mice, the tumor burden in bone was markedly decreased compared with that in untreated mice (Sasaki *et al.* 1995). These findings and a similar study in rats (Hall & Stoica 1994) suggest that reduced growth factor release from bone due to bisphosphonate-induced inhibition of osteoclastic bone resorption may impair metastatic breast cancer growth in bone. Of note, in some experiments, we observed that metastases of MDA-231 cells to liver and adrenal gland were increased in bisphosphonate-treated mice. Similar experimental findings have been reported by Kostenuik *et al.* (1993) and Stearns and Wang (1996). However, the clinical significance of these observations for humans with metastatic cancer has yet to be elucidated.

To further test our hypothesis that tumor cell metastasis and proliferation are enhanced by the products of bone resorption, we increased bone resorption by injecting IL-1 locally over the calvaria of nude mice for 3 days according to the methods described by Boyce *et al.* (1992). Breast cancer cells were inoculated into the left cardiac ventricle the following day, and the mice were examined for the development of cancer metastases in the calvariae at 4 weeks after cell inoculation. Cancer cells rarely metastasize spontaneously to calvariae in this model and we speculated that this was due to a low basal level of bone turnover in calvariae compared with other bones. Metastatic tumor deposits were clearly visible in the calvariae of IL-1-treated mice (Sasaki *et al.* 1994), and radiologic and histologic examination revealed that these were osteolytic. In contrast, no metastatic tumor deposits were detected in calvariae of phosphate-buffered saline (PBS)-treated mice. Furthermore, treatment of mice with risedronate prior to IL-1 injections to suppress the IL-1-induced increase in bone turnover significantly reduced metastasis formation (Sasaki *et al.* 1994).

To further study the effects of bone-derived factors on tumor cell growth, we added the supernatants from resorbing neonatal mouse calvariae to cultures of breast cancer cells and found that they strongly increased breast cancer cell proliferation (Yoneda *et al.* 1995). However, no stimulation of breast cancer cell proliferation was observed when bone resorption was inhibited *in vitro* by risedronate. Furthermore, the growth-stimulating effects of the resorbing bone culture supernatants on the tumor cells was markedly impaired by neutralizing antibodies to IGF-I receptors (Yoneda *et al.* 1995).

Metastatic cancer cell growth might also be affected by osteoblasts in bone. For example, the culture supernatants of osteoblasts increase both chemotactic migration and matrix metalloproteinase production by breast cancer cells *in vitro* (Giunciuglio *et al.* 1995). However, further studies are needed to fully examine the role of osteoblasts in cancer colonization in bone.

General cancer cell properties involved in bone metastasis

Cell adhesion molecules have been shown to have key roles in several critical steps involved in cancer cell

invasion and metastasis. In normal cells they mediate cell-to-cell and cell-to-substratum communications, and cancer cells may decrease or increase their expression of CAMs to assist them to leave the primary site and to attach and proliferate at a fertile metastatic site (Albelda & Buck 1990).

Integrins are the most abundantly expressed CAMs (Haynes 1992). They have been implicated in cancer dissemination (Juliano & Varner 1993) by mediating cancer cell attachment to vascular endothelial cells and to underlying matrix proteins, such as laminin and fibronectin (Albelda & Buck 1990). Expression of the $\alpha_v\beta_3$ integrin (vitronectin receptor) on the surface of human melanoma cells increases when they bind to and invade the basement membrane matrix, matrigel (Seftor *et al.* 1992), and neutralizing antibodies to $\alpha_v\beta_3$ integrins inhibit tumor growth and invasion *in vivo* (Brooks *et al.* 1994). However, their role in bone metastasis has not been studied, as yet.

Laminin is a major component of basement membrane that has been implicated in malignancy (Menard *et al.* 1997). We have carried out two studies that suggest a role for laminin in bone metastasis. A multimeric anti-laminin peptide, YIGSR, reduced the growth of osteolytic metastases of a human melanoma cell line, A375 (Nakai *et al.* 1992), and of a B-cell lymphoma cell line, MH-95 (Michigami *et al.* 1999) in nude mice innoculted with these tumors cells. This peptide has been reported to inhibit angiogenesis (Iwamoto *et al.* 1996). Thus, these studies implicate angiogenesis in the development of bone metastases.

E-cadherin (Uvomorulin) is a 120 kDa cell surface glycoprotein involved in calcium-dependent epithelial cell-cell adhesion which appears to play a suppressive role in cancer invasion and metastasis (Takeichi 1993). E-cadherin expression in cancer cells is reversibly modulated according to culture conditions *in vitro* and environmental factors *in vivo* (Mareel *et al.* 1991*b*). Its expression in human tumors correlates inversely with breast cancer metastasis (Oka *et al.* 1993) and is increased in sub-populations of MCF-7 breast cancer cells with reduced invasiveness, but is undetectable in the highly invasive MDA-MB-231 breast cancer cells (Sommers *et al.* 1991). Most studies to date have examined only the inhibitory effects of E-cadherin on invasiveness of tumor cells at the primary site and few have examined the possible role of E-cadherin in cancer metastasis to distant organs, including bones. To examine this question, we stably transfected MDA-MB-231 cells with E-cadherin and found markedly fewer osteolytic metastases in nude mice inoculated with the transfected cells compared with controls (Mbalaviele *et al.* 1996), suggesting that increased cell-cell adhesiveness may reduce their capacity to grow in bone.

In a murine myeloma model associated with massive osteoclastic bone destruction, we have found that the cell-cell interactions between marrow stromal cells and myeloma cells mediated via VCAM-1 and (4(1 integrin VCAM, respectively, are critical for myeloma cell production of osteoclast-activating factor(s) (Michigami *et al.* 1997). Disruption of these interactions could be an effective therapeutic intervention for the treatment of the devastating bone destruction seen typically in myeloma.

Organ-selective adherence of cancer cells

A number of investigators have attempted to examine organ selectivity of malignant cells. For example, Kieran & Longnecker (1983) and Netland & Zetter (1984) showed that ^{57}Cr-labeled cancer cells bound selectively to fresh cryostat sections of particular host organs. In more convincing studies, Nicholson (1988) showed that cells can exhibit preference for specific sites. Following injection of tumour cells into the tail vein of mice, he removed cells that formed brain or lung metastases form these sites and repeatedly reinjected them into other mice. He thus established two sub-clonal cell lines of the B16 murine melanoma. One spread preferentially to brain and adhered selectively to brain-derived endothelial cells, while another sub-clone metastasized preferentially to the lungs. Furthermore, Haq *et al.* (1992) found that the rat Dunning prostate carcinoma cell line that disseminates to bone adheres preferentially to cultures of bone marrow stromal cells enriched for endothelial cells.

Highly invasive cancer cells produce large amounts of MMPs that comprise a family of at least eight zinc-dependent endopeptidases with related structures, but different substrate specificities. Increased expression of MMPs correlates with the development of invasion and metastasis in human breast, colon, stomach, thyroid, lung and liver cancers (Seftor *et al.* 1992, Zucker *et al.* 1993). It is likely that individual cancer cells utilize several MMPs as well as other classes of destructive enzymes to cross the various tissue boundaries they encounter as they invade and metastasize.

We have examined MMP expression by MDA-MB-231 and found that when the cells were cultured on plastic they produce 92 kDa (MMP-9) and 72 kDa (MMP-2) MMPs in latent forms. However, when they were cultured on bone extracellular matrix laid down by osteoblasts the cells released active forms of both these MMPs, while others cultured on laminin, fibronectin, type I collagen, matrigel and poly-L-lysine released latent forms of MMPs (Yoneda *et al.* 1997).

Cancer invasiveness and metastatic capacity is determined not only by expression levels of MMPs, but also by those of the corresponding tissue inhibitors of matrix metalloproteinases (TIMPs), at least two of which

are distributed ubiquitously (Liotta 1992). TIMPs function as metastasis suppressors, and the invasive capacity of cancer cells is likely to depend on the balance between MMP and TIMP production. Transfection of tumor cells with the TIMP-2 gene partially suppressed their invasion and metastasis in animals, and injections of recombinant TIMP-2 blocked metastasis of tumor cells (DeClerck *et al.* 1992). We transfected MDA-MB-231 cells with TIMP-2 cDNA and in a preliminary experiment observed that mice inoculated with these cells had fewer osteolytic metastases than mice inoculated with MDA-MB-231 cells transfected with the empty vector (Yoneda *et al.* 1997).

PTHrP and bone metastasis

PTHrP is produced by a variety of normal tissues, including breast, skin, placenta, uterine and vascular smooth muscle (Philbrick *et al.* 1996), and is also produced by many types of malignant tumors both at the primary site and at sites of metastasis (Danks *et al.* 1989, Asa *et al.* 1990, Dunne *et al.* 1993). In a study examining its expression by breast cancer cells, PTHrP was detected immunohistochemically in 56% of 155 primary breast tumors from normocalcemic women and its expression correlated with the development of bone metastases (Bundred *et al.* 1992). However, PTHrP expression in primary breast tumors does not appear to correlate with standard prognostic factors, recurrence or survival (Southby *et al.* 1990, Bundred *et al.* 1992, Liapis *et al.* 1993). Its expression by breast cancer cells appears to increase when the tumor cells metastasize to bone. For example, it has been detected by immunohistochemistry (Powell *et al.* 1991, Kohno *et al.* 1994) and *in vivo* hybridization (Vargas *et al.* 1992) in 80-90% of breast cancer metastases in bone compared with only 38% in lung (Kohno *et al.* 1994) and 17% (Powell *et al.* 1991) in other non-bone sites. These findings suggest that PTHrP expression by breast cancer cells increases when they metastasize to bone and support a major potential role for tumor-produced PTHrP to mediate the increased bone resorption around osteolytic breast metastases.

In view of these clinical observations, we have used the nude mouse model of bone metastasis and MDA-MB-231 cells which produce low amounts of PTHrP constitutively *in vitro* to further examine the role of PTHrP in the development of bone metastases. We increased PTHrP production by these cells by transfecting them with the cDNA for human preproPTHrP and inoculated them into nude mice. This resulted in an increase in the number of osteolytic metastases *in vivo* in the mice bearing the transfected cells compared with controls (Guise *et al.* 1994). Furthermore, treatment of mice with monoclonal antibodies directed against the 1-34 region of PTHrP prior to inoculation with parental MDA-MB-231 cells dramatically reduced the number and size of osteolytic lesions compared with controls (Guise *et al.* 1996). Treatment with the PTHrP antibody also decreased the rate of progression of established osteolytic metastases compared with controls (Yin *et al.* 1995).

These data strongly suggest that PTHrP expression by breast cancer cells promotes the development and progression of metastases in bone, but they do not provide an explanation for the clinical observation that PTHrP expression by breast cancer cells increases when the cells metastasize to bone. Several candidate factors have been identified that could promote increased release of PTHrP from breast cancer cells in bone. For example, epidermal growth factor (EGF) stimulates PTHrP expression by a keratinocyte (Allinson & Drucker 1992) and a mammary epithelial cell line (Sebag *et al.* 1994), while TGFα enhances PTHrP expression in a human squamous cell carcinoma of the lung (Burton *et al.* 1990). IL-6, tumor necrosis factor (TNF), IGF-I and IGF-II increased the production of PTHrP *in vitro* by a human squamous cell carcinoma (Rizzoli *et al.* 1994). TGFß, which is present in high concentrations in bone matrix (Hauschka *et al.* 1986) and is expressed by some breast cancers (Dublin *et al.* 1993) and cancer-associated stromal cells (van Roozendaal *et al.* 1995), has been shown to enhance secretion of PTHrP in a renal and squamous cell carcinoma (Kiriyama *et al.* 1992, Merryman *et al.* 1994). TGFß is stored in bone and is released and activated during osteoclastic bone resorption (Pfeilschifter & Mundy 1987). It increases PTHrP expression by MDA-MB-231 cells *in vitro* and thus could potentiate the development of bone metastases by increasing PTHrP production by these cells in bone metastases.

To examine this possible role for TGFβ, we transfected MDA-MB-231 cells with a cDNA encoding a TGFβ type II receptor lacking a cytoplasmic domain (TβRIIΔcyt) and inoculated the cells into the left ventricle of nude mice. TGFβ binds to this receptor, but signal transduction is not initiated and, thus, the receptor acts in a dominant-negative fashion to block the biologic effects of TGFβ. PTHrP secretion did not increase in response to TGFβ in stable clones expressing TβRIIΔcyt compared with controls of untransfected MDA-MB-231 cells or those transfected with the empty vector. Mice inoculated with MDA-MB-231 cells expressing TβRIIΔcyt had fewer and smaller osteolytic lesions than control mice given parental or empty vector-transfected cells (Yin *et al.* 1999). Reversal of the dominant-negative signaling blockade by expression of a constitutively active TGFβ type I receptor in the breast cancer cells increased tumor production of PTHrP, caused marked enhancement of osteolytic bone metastasis (Figs 1 and 2) and decreased survival. To determine if the effects of TGFβ to increase bone metastases were mediated by PTHrP, the MDA-MB-

Table 2 Factors produced by tumor cells that stimulate bone formation

Bone-derived growth factor (BDGF, B2 microglobulin
Bone morphogenetic proteins (BMPs)
Endothelin 1
Fibroblast growth factors (FGF acidic and basic)
Insulin-like growth factors (IGF-I and II)
Interleukin-1 (IL-1)
Macrophage-derived growth factor (MDGF)
Platelet-derived growth factor (PDGF)
Prostaglandins
Transforming growth factor β (TGFβ)
Tumor necrosis factor α (TNFα)
Tumor necrosis factor β (Lymphotoxin)
Urokinase (Urinary Plasminogen Activator, uPA)

231 cells which expressed the dominant-negative TGFβ type II receptor were transfected with the cDNA for PTHrP. This resulted in a marked increase in tumor PTHrP production and accelerated bone metastases. To determine if the effects of TGFβ to promote bone metastases were mediated by PTHrP, the MDA-MB-231 cells which expressed the dominant-negative TGFβ type II receptor were transfected with the cDNA for PTHrP (Yin *et al.* 1999). This resulted in constitutive tumor PTHrP production and accelerated bone metastasis. These data demonstrate an important role for TGFβ in the development of breast cancer metastasis to bone, via the TGFβ receptor-mediated signaling pathway in tumor cells, and suggest that the bone destruction is mediated by PTHrP. Thus, these findings support the hypothesis that TGFβ released from bone during metastasis-stimulated resorption maintains an up-regulatory loop in which it promotes PTHrP secretion by breast cancer cells. This in turn promotes more osteoclastic bone resorption and more release of TGFβ from bone thus inducing further tumor growth and bone destruction.

Although loss of TGFβ receptor function (Markowitz *et al.* 1995) or its signaling molecules (Eppert *et al.* 1996, Hahn *et al.* 1996, Takaku *et al.* 1998, Zhu *et al.* 1998) has been associated with malignant progression (Massagué

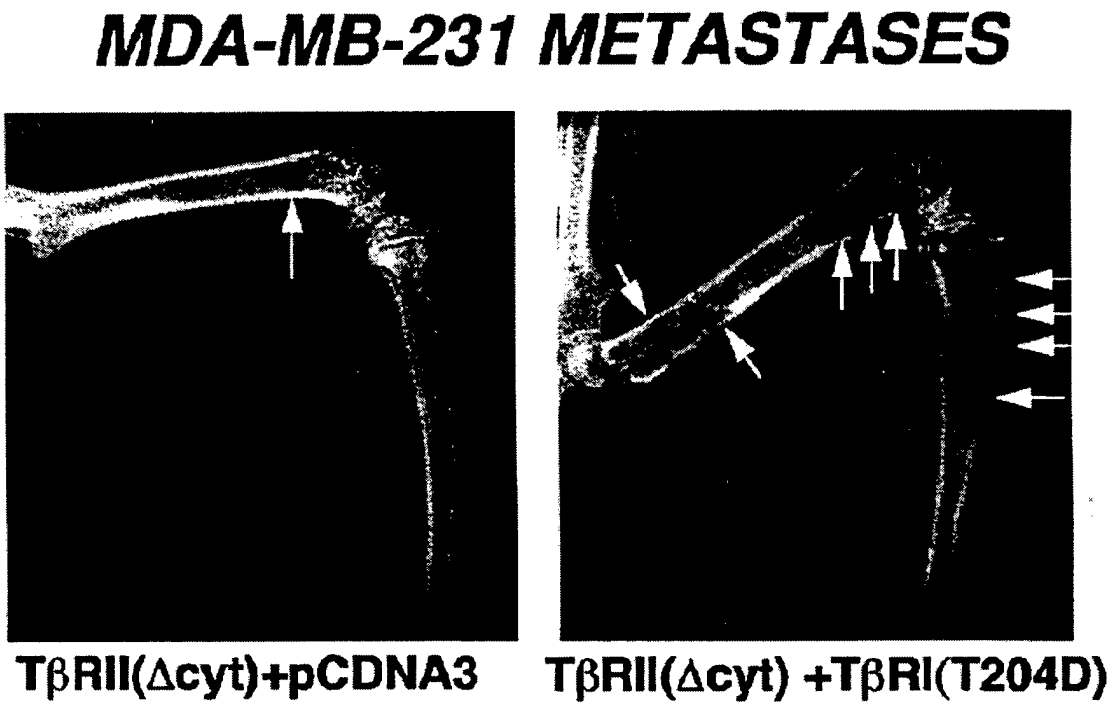

Figure 1 Representative radiographs of hindlimbs from nude mice bearing MDA-231 breast cancer cells transfected with a dominant-negative TGFß receptor (TßRII_cyt) + an empty vector (pcDNA3.1zeo; pcDNA3) or with a TßRII_cyt + a constitutively active TGFß receptor (TßRII_cyt) 28 days after tumor inoculation. Increased numbers of osteolytic lesions (indicated by the arrows) were seen in the mice inoculated with tumor cells transfected with the TßRII_cyt + TßRII_cyt transfected cells. Reproduced with permission from Authors and date; *Journal of Clinical Investigation* vol, pp.

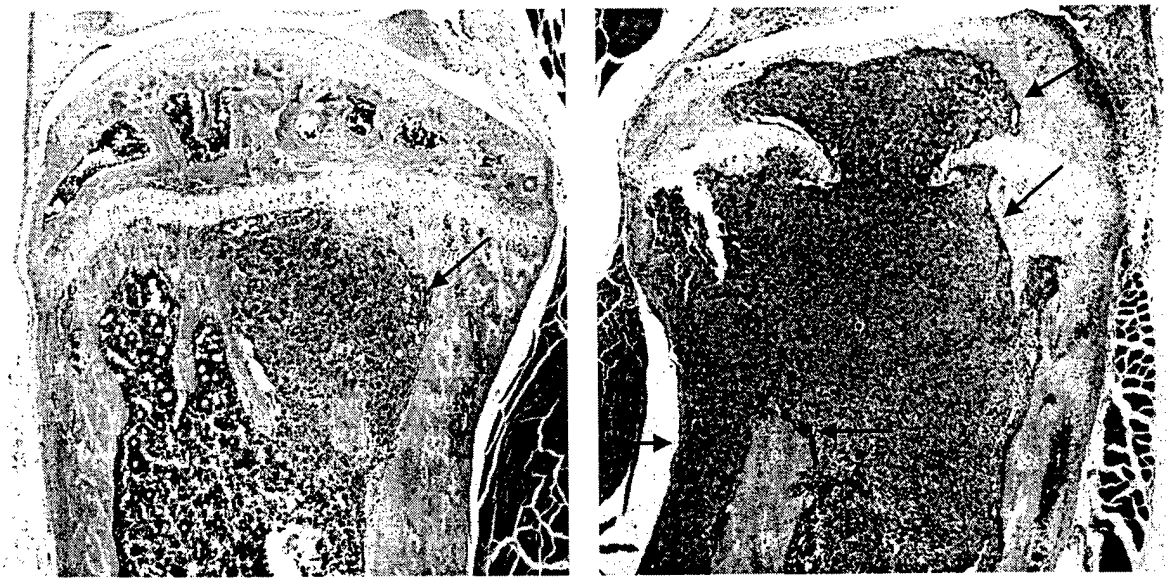

Figure 2 Bone histology from the proximal tibiae of representative mice bearing either MDA-231 cells transfected with either TßRII_cyt + TßRI(T204D) or TßRII_cyt + pcDNA3.1zeo (pcDNA3). Tumor (arrows) filled much of the marrow cavity and replaced normal cellular elements in mice bearing TßRII_cyt + TßRI(T204D) tumors (right). There was significant loss of both cortical and trabecular bone in this group, and tumor has eroded through the growth plate of the tibia of this mouse. In contrast, sections from mice bearing control TßRII_cyt + pcDNA3.1zeo tumors (left) had small foci of tumor in the marrow cavity (arrow) with little bone destruction, as evidenced by intact trabecular and cortical bone. Reproduced with permission from Authors and date; *Journal of Clinical Investigation* vol, pp.

1998), there is growing evidence that TGFβ may enhance tumor growth and invasion. For example, TGFβ induces an epithelial-mesenchymal transdifferentiation and an invasive phenotype (Miettinen *et al.* 1994, Caulin *et al.* 1995). Oft *et al.* (1998), using the same dominant-negative TGFβ type II receptor approach, demonstrated that TGFβ blockade decreased invasion and metastases in a mouse colon carcinoma and that several human carcinoma cell lines lost *in vitro* invasiveness when treated with neutralizing TGFβ antibodies. Thus, it appears that TGFβ signaling is required for both induction and maintenance of invasiveness *in vitro* and metastasis during late-stage tumorigenesis.

Metastatic prostate cancer

Prostatic cancer, like breast cancer, has a distinct predilection for metastasis to and growth within bone. Up to 70% of patients with advanced prostatic cancer have bone metastases, and most of these are osteoblastic. Metastases are found most frequently in lumbar vertebrae and pelvic bones following retrograde spread via Barton's vertebral venous plexus, and bone is the second commonest metastatic site after regional lymph nodes (Galasko 1981). However, diffuse skeletal involvement is relatively common, as are mixed osteoblastic and osteolytic lesions. The cancer cells appear to stimulate new bone formation by causing osteoblasts on fully calcified, 'quiescent' bone surfaces to lay down new matrix without preceeding resorption and also by stimulating osteoblast precursors in the bone marrow to proliferate and lay down new bone matrix between pre-existing bone trabeculae (Valentin *et al.* 1980). Osteoblastic metastases are typically 'hot' on bone scan and, although osteosclerotic, may result in vertebral collapse and paraplegia because much of the new bone is woven with intrinsically low strength and because there may be concomitant osteolysis.

Normal and malignant prostatic cells express a host of growth factors (see Table 2) and some of their receptors (reviewed by Koutsilieris 1995), some of which, such as TGFβ, BMPs, IGFs and FGFs, can stimulate osteoblast proliferation, while others, such as PTHrP, platelet-derived growth factor (PDGF) and TGFβ, stimulate bone resorption (Iwamura *et al.* 1993). Furthermore, bone marrow stromal cells (Chackal-Roy *et al.* 1989) and, in particular, cells in the osteoblast lineage (Gleave *et al.* 1992), produce factors which are mitogenic for prostatic

Table 2 Factors produced by tumor cells that stimulate bone formation

Bone-derived growth factor (BDGF, B2 microglobulin)
Bone morphogenetic proteins (BMPs)
Endothelin 1
Fibroblast growth factors (FGF acidic and basic)
Insulin-like growth factors (IGF-I and II)
Interleukin-1 (IL-1)
Macrophage-derived growth factor (MDGF)
Platelet-derived growth factor (PDGF)
Prostaglandins
Transfroming growth factor β (TGFβ)
Tumor necrosis fator α (TNFα)
Tumor necrosis factor β (Lymphotoxin)
Urokinase (Urinary Plasminogen Activator, uPA)

cancer cells, indicating that there may be bi-directional interactions favoring the growth of tumor cells and osteoblasts in close proximity to one another.

In addition to these growth factors, prostatic cancer cells produce urokinase-type plasminogen activator (uPA) and endothelin-1 (Nelson *et al.* 1995) which may enhance the growth of osteoblasts at the metastatic site. Indeed, plasma levels of endothelin-1 are elevated in patients with metastatic prostatic cancer (Nelson *et al.* 1995). uPA appears to stimulate osteoblast proliferation by hydrolysing IGF-binding proteins and thus activating the growth factors (Koutsilieris *et al.* 1993). Furthermore, transfection of rat prostate cancer cells with full-length uPA cDNA promotes the growth of the tumor cells themselves and causes earlier development of metastases after intracardiac injection of the transfected cells compared with controls (Koutsilieris *et al.* 1993).

Although osteoblastic bone metastases are common in advanced prostate and breast cancer, the pathogenesis of the increased bone formation remains poorly understood, in part because there are few models of metastasizing osteosclerotic tumors. During study of the effects of a human breast cancer cell line, ZR-75-1, in our bone metastasis model, we discovered that these tumor cells cause osteoblastic metastases. We compared the effects on bone of these tumor cells with those of the human breast cancer line, MDA-MB-231 (MDA-231), which causes osteolytic metastases. ZR-75-1, MDA-231 or vehicle control (PBS) were inoculated into the left cardiac ventricle of nude mice. Histomorphometric analysis of long bones from these animals demonstrated significantly greater bone volume in ZR-75-1-bearing mice compared with those bearing MDA-231 or treated with PBS. There was no significant difference in tumor volume between ZR-75-1- and MDA-231-treated animals. Osteoclast number per long bone section was greatest in MDA-231-bearing mice, but there was no significant difference between ZR-75-1 and PBS (Guise & Mundy 1998). To determine the mechanism responsible for the increased new bone formation in the ZR-75-1-bearing mice, ZR-75-1 cells were screened for factors known to stimulate new bone formation and compared with MDA-231. Of these factors (TGFβ-1 and -2, BMP-2, -3, -4, -6, IGF-I and -II, prostate-specific antigen (PSA), uPA, and endothelin-1 (ET-1)), ET-1 was the only potential osteoblast-stimulating factor produced in greater amounts *in vitro* by ZR-75-1 cells compared with MDA-231 cells. PTHrP was produced by MDA-231 and not ZR-75-1 cells. To investigate whether ET-1 plays a role in the new bone formation stimulated by ZR-75-1 cells, the effect of ET-1 and ZR-75-1 conditioned medium was tested on new bone formation in neonatal mouse calvarial organ cultures. ZR-75-1 conditioned medium and synthetic ET-1 each stimulated osteoblast proliferation and new bone formation *in vitro* in a dose-dependent manner, comparable to that stimulated by BMP-2 or FGF-2. In contrast, conditioned media from MDA-231 cells had no effect on new bone formation. New bone formation caused by both ZR-75-1 conditioned medium and ET-1 was inhibited by BQ-123, an endothelin A receptor antagonist. Finally, we have identified two other human breast cancer cell lines which secrete ET-1 and cause osteoblastic metastases *in vivo*. The preliminary data suggest that the effects of ET-1 on new bone formation are mediated via the endothelin A receptor.

The production of osteoclast-stimulating factors by prostate cancer cells could account for the osteolysis seen in some metastases. The expression level of these by tumor cells relative to that of osteoblast-stimulating factors is likely to determine whether individual metastases are osteolytic, osteoblastic or mixed. Recent studies have shown that PSA, a serine protease, homologous to the kallikrien family of proteases (Riegman *et al.* 1989) can cleave PTHrP and completely abolish its ability to stimulate cAMP production (Cramer *et al.* 1996). Thus, although PTHrP is produced by many prostate cancers, it could be inactivated in bone by PSA, thus allowing an osteoblast response to predominate in most metastases.

Summary

Clinically overt bone metastases are common in patients with advanced cancer and their presence is typically accompanied by a grave prognosis. Recent studies have identified a number of factors that regulate the proliferation and activity of metastatic cells and their interaction with bone cells after they have seeded in bone. However,

despite advances in our understanding of the close interaction that takes place between cancer cells and bone cells, the specific molecular mechanisms by which cancer cells spread to and destroy bone or stimulate new bone formation remain poorly understood. Furthermore, therapeutic agents with proven efficacy to prevent or reverse metastatic bone disease have yet to be developed. Recent studies indicating that bisphosphonate therapy may have beneficial direct and indirect effects on tumor cell growth in bone suggest that the poor prognosis associated with metastatic bone disease could be ameliorated with intervention therapy particularly if it can be demonstrated that therapy given early in the course of the disease prevents the development of metastases. Many of the factors produced in excess by tumor cells in bone are likely to be released locally in much lower concentrations by normal cells within bone marrow and to be involved in the regulation of bone remodeling. Thus, study of the effects of cancer cells on bone should not only benefit patients with cancer, but also improve our understanding of the regulation of bone turnover in normal and disease states.

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Breast Cancer Cells Interact with Osteoblasts to Support Osteoclast Formation*

RACHEL J. THOMAS, THERESA A. GUISE, JUAN JUAN YIN, JAN ELLIOTT, NICOLE J. HORWOOD, T. JOHN MARTIN, AND MATTHEW T. GILLESPIE

St. Vincent's Institute of Medical Research and The University of Melbourne, Department of Medicine (R.J.T., J.E., N.J.H., T.J.M., M.T.G.) St. Vincent's Hospital, Fitzroy, Victoria 3065, Australia; and The Department of Medicine, Division of Endocrinology at the University of Texas Health Science Center, (T.A.G., J.J.Y.) San Antonio, Texas 78284

ABSTRACT

Breast cancers commonly cause osteolytic metastases in bone, a process that is dependent upon osteoclast-mediated bone resorption. Recently the osteoclast differentiation factor (ODF), better termed RANKL (receptor activator of NF-κB ligand), expressed by osteoblasts has been cloned as well as its cognate signaling receptor, receptor activator of NFκB (RANK), and a secreted decoy receptor osteoprotegerin (OPG) that limits RANKL's biological action. We determined that the breast cancer cell lines MDA-MB-231, MCF-7, and T47D as well as primary breast cancers do not express RANKL but express OPG and RANK. MCF-7, MDA-MB-231, and T47D cells did not act as surrogate osteoblasts to support osteoclast formation in coculture experiments, a result consistent with the fact that they do not express RANKL. When MCF-7 cells overexpressing PTH-related protein (PTHrP) were added to cocultures of murine osteoblasts and hematopoietic cells, osteoclast formation resulted without the addition of any osteotropic agents; cocultures with MCF-7 or MCF-7 cells transfected with pcDNAIneo required exogenous agents for osteoclast formation. When MCF-7 cells overexpressing PTHrP were cultured with murine osteoblasts, osteoblastic RANKL messenger RNA (mRNA) levels were enhanced and osteoblastic OPG mRNA levels diminished; MCF-7 parental cells had no effect on RANKL or OPG mRNA levels when cultured with osteoblastic cells. Using a murine model of breast cancer metastasis to bone, we established that MCF-7 cells that overexpress PTHrP caused significantly more bone metastases, which were associated with increased osteoclast formation, elevated plasma PTHrP concentrations and hypercalcaemia compared with parental or empty vector controls. (*Endocrinology* **140:** 4451–4458, 1999)

IT HAS LONG been recognized that breast cancers have the ability to invade and grow as metastases in bone (1). Some breast cancer cell lines have been shown to induce osteolytic lesions in animal models that mimic the metastatic process in clinical breast cancer (2, 3), but the factors favoring breast cancer growth in bone remain to be resolved. We and others have established that breast cancer cell lines and primary breast cancers have a number of phenotypic properties in common with bone cells, for example in their expression of calcitonin receptors, bone sialoprotein, cathepsin K, and osteopontin (4, 5), and we have proposed that these properties may contribute to a breast cancer's capacity to establish and grow in bone (4). Additionally, the production of bone-resorbing factors (such as PTH-related protein, PTHrP) by breast cancer cells may be among the properties that contribute to this (6, 7), although it is likely that other features also favor growth in skeletal tissue.

Most breast cancer metastases in bone form osteolytic lesions, in contrast with bone secondaries from prostate cancer which are osteosclerotic (1). Although it has been postulated that bone destruction by breast cancer is mediated directly by tumor cells (1), most evidence indicate that breast cancer-induced bone destruction is mediated by the osteoclast. Support for the latter include 1) breast cancers express cytokines (such as IL-1, IL-6, LIF, prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNFα) and PTHrP) which can influence osteoclast formation (1); 2) histologic analysis of osteolytic lesions reveal tumor adjacent to osteoclasts resorbing bone; 3) and use of bisphosphonates, potent inhibitors of osteoclastic bone resorption, in women with breast cancer metastases to bone results in reduced skeletal morbidity (8, 9).

The process of mouse osteoclast formation can be studied *in vitro* by culturing bone marrow culture cells and by coculture of osteoblastic stromal cells with hematopoietic cells, both of which result in the formation of *bona vide* osteoclasts in response to various osteotropic factors; the osteoclasts stain for tartrate-resistant acid phosphatase (TRAP), are multinucleated, exhibit calcitonin receptors (CTR) and most importantly can resorb bone (10, 11). In both systems, stromal osteoblasts support osteoclast formation from precursors of hematopoietic origin.

Recently the stromal cell-derived osteoclast differentiation factor (ODF) or osteoprotegerin ligand (OPGL) has been identified, and a soluble form of the molecule in combination with M-CSF can generate osteoclasts from hematopoietic cells in the absence of osteoblastic stromal cells (11–14): it was also identified by its ability to induce NFκB and apoptosis of T-cells as receptor activator of NF-κB ligand (RANKL) and tumor necrosis factor-related activation-induced cytokine (TRANCE), respectively (15–17). For simplicity, will we adopt its nomenclature as RANKL, which represents the

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Address all correspondence and requests for reprints to: Dr. Matthew T. Gillespie, St. Vincent's Institute of Medical Research, 41 Victoria Parade, Fitzroy 3065, Victoria, Australia. E-mail: m.gillespie@medicine.unimelb.edu.au.

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functional property that has been described for this molecule. RANKL is a member of the tumor necrosis factor (TNF) family and is a membrane-bound molecule, there is no evidence for an alternatively spliced form of this molecule, although the molecule is shed from the plasma membrane as a result of protease action from HEK293 cells overexpressing RANKL/OPGL (12). Two receptors for RANKL have been proposed. The first, which aided the identification RANKL was osteoprotegerin (OPG), also reported as osteoclastogenesis inhibitory factor (OCIF) (18, 19). OPG is a secreted TNF receptor family member and has a relatively wide distribution. Overexpression of OPG in mice resulted in osteopetrosis (18). Conversely, mice deficient in OPG demonstrate osteoporosis and calcification of the aorta (20). In agreement with the phenotypes of mice with altered OPG production, recombinant OPG inhibited osteoclast formation in cocultures of mouse osteoblastic cells and hematopoietic cells (12, 13). The ability of OPG to bind to RANKL and limit the biological actions of RANKL suggested that OPG may function as a decoy receptor (12, 13, 19, 21). The second, putative receptor, and likely responsible for signaling RANKL biological actions, appears to be receptor activator of NFκB (RANK) (15), although other hitherto unrecognized receptors of the TNF-receptor family may have similar capabilities. In this study, we have investigated the expression of RANKL, OPG, and RANK in breast cancer cell lines and primary tumors. We have also investigated whether breast cancers can directly support or indirectly influence osteoclastogenesis using the murine coculture system and we have demonstrated the ability of breast cancer cells to regulate RANKL expression of stromal osteoblasts. Finally, we have assessed the *in vivo* osteolytic potential of breast cancer cell lines which differ in their osteoclast-inductive capacity *in vitro*.

Materials and Methods

Animals, cell lines, and drugs

Male newborn (0–1 day old) C57/BL6J mice were purchased from Monash University Animal Services Centre (Clayton, Australia). $1\alpha,25(OH)_2$ D_3 was purchased from Wako Pure Chemical Co. (Osaka, Japan), and PGE_2 was obtained from Sigma (St. Louis, MO).

Coculture system

Osteoblastic cells were prepared from the calvaria of newborn mice by sequential digestion with 0.1% collagenase (Worthington Biochemical Corp. Co., Freefold, Australia) and 0.2% dispase (Godo Shusei, Tokyo, Japan). Bone marrow was obtained from the femur and tibia of adult C57BL6J male mice. Osteoblastic cells and/or breast cancer cells were cocultured with spleen or marrow cells as previously described (22, 23). The expression of calcitonin receptors was also assessed by autoradiography using [^{125}I]-salmon calcitonin as described (22) and resultant cells from coculture experiments were tested for their ability to resorb bone (24).

Tissue specimens

Twelve breast lesions were collected from patients undergoing resective surgery at St. Vincent's Hospital. The tissues were immediately placed on dry ice and stored at −70 C. The tissues examined were all infiltrating ductal carcinomas with two tumors containing a ductal carcinoma *in situ* component.

RNA extraction, complementary DNA (cDNA) synthesis, and PCR

Total RNA extraction from the tissues, cDNA synthesis, and PCR were performed as described (25). Oligonucleotides were designed to amplify and detect human RANKL (GenBank accession number AF019047), murine RANKL (GenBank accession number AF019048), human OPG (GenBank accession number U94332), murine OPG (GenBank accession number U94331) and human RANK (GenBank accession number AF018253) (Table 1). Finally, for glyceraldehyde phosphate dehydrogenase (GAPDH) primers used have been published previously GAPDH-1, GAPDH-2, GAPDH-3, and GAPDH-4 (25). The specificity of the products was confirmed by Southern blot detection using a ^{32}P-labeled internal oligonucleotide probe, as previously described (25) or by nucleic acid sequencing of amplified products. Bound probe was detected by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA).

Stable transfection of MCF-7 cells

Full-length prepro-PTHrP (1–139) was cloned into the mammalian expression plasmid pcDNAIneo and was subsequently introduced into the MCF-7 cell lines by calcium phosphate precipitation (26). MCF-7 cells were also transfected with pcDNAIneo vector alone to act as a further control. Single clones were isolated by limiting dilution in the presence of the selective marker G418 (Sigma, St. Louis, MO). Clones were screened by measuring secreted PTHrP in serum-free 24 h conditioned media and by messenger RNA (mRNA) levels. The levels of PTHrP secreted by these cells in conditioned media for 48 h, corrected for 10^5 cells, were 1609 ± 171 pmol/liter (MCF-7 PTHrP 139c), 4.3 ± 0.3 pmol/liter (MCF-7 pcDNAIneo, EV) and 1.8 ± 0.4 pmol/liter (MCF-7).

TABLE 1. Oligonucleotides used in the amplification and detection of RT-PCR products

Oligo name	Species/orientation	Nucleotides	Sequence
RANKL-1	Murine Sense	381–400	5′-ATCAGAAGACAGCACTCACT-3′
RANKL-2	Murine Antisense	1106–1130	5′-ATCTAGGACATCCATGCTAATGTTC-3′
RANKL-6	Human Sense	569–586	5′-TCTAACCATGAGCCATCC-3′
RANKL-11	Murine Sense	1040–1055	5′-CGGATCAAGATGCGAC-3′
RANKL-15	Human Sense	533–555	5′-TGGATCACAGCACATCAGAGCAG-3′
RANKL-16	Human Antisense	1065–1088	5′-TGGGGCTCAATCTATATCTCGAAC-3′
OPG-1	Murine Sense	778–795	5′-ACCAAAGTGAATGCCGAG-3′
OPG-2	Murine Antisense	1305–1323	5′-AAGAAACAGCCCAGTGACC-3′
OPG-3	Murine Antisense	1021–1040	5′-TTTCTCGTTCTCTCAATCTC-3′
OPG-4	Human Sense	85–106	5′-GGGGACCACAATGAACAAGTTG-3′
OPG-5	Human Antisense	472–493	5′-AGCTTGCACCACTCCAAATCC-3′
OPG-6	Human Antisense	291–313	5′-GCTGTCTGTGTAGTAGTGGTCAG-3′
RANK-10	Human Sense	753–776	5′-GGGAAAGCACTCACAGCTAATTTG-3′
RANK-11	Human Antisense	1181–1205	5′-GCACTGGCTTAAACTGTCATTCTCC-3′
RANK-15	Human Antisense	854–873	5′-GCTGACCAAAGTTTGCCGTG-3′

In vivo experiments

Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Female nude mice 4–6 weeks of age were housed in laminar flow isolated hoods with 12-h light, 12-h dark cycle. Water supplemented with vitamin K and autoclaved mouse chow were provided *ad libitum*.

Whole blood samples for ionized calcium concentration were obtained by retro-orbital puncture under metofane anesthesia. Blood samples for PTHrP measurement were similarly obtained and collected on ice in vacutainer tubes containing EDTA (Becton Dickinson and Co., NJ) and 400 IU/ml aprotinin (Sigma, St. Louis, MO).

Tumor inoculation into the left cardiac ventricle was performed while the mice were anesthetized with a ketamine/xylazine mixture and positioned ventral side up based on a modification of Arguello (27). Because MCF-7 cells are estrogen dependent, mice were implanted with a 60-day slow release 17β-estradiol pellet (0.5 mg, 3 mm; Innovative Research of America, Sarasota, FL) before tumor inoculation. 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 correct position in the left cardiac ventricle.

Bone metastasis

Mice were inoculated with tumor cell suspensions of MCF-7 PTHrP 139c, MCF-7 pcDNAIneo or MCF-7 cells into the left cardiac ventricle (n = 5 per group) on day 0. Baseline radiographs and body weights as well as blood for Ca^{2+} and plasma PTHrP concentrations were obtained at this time. Radiographs were taken on day 21 and then weekly until they were killed. At the time mice were killed, blood was collected for Ca^{2+} and PTHrP measurement, and all bones and soft tissues were harvested and fixed in formalin for histologic analysis. Autopsy was performed on all mice, and those with tumor in the chest were excluded from analysis, as this indicated that part or all of the tumor inoculum did not properly enter the left cardiac ventricle. This experiment was performed twice with similar results obtained.

Ca^{2+} measurement

Ca^{2+} concentrations were measured in whole blood using a Ciba Corning, Inc. 634 ISE Ca^{2+}/pH analyzer (Medfield, MA) and adjusted using the internal algorithm of the instrument to pH 7.4. Samples were run in duplicate and the mean value recorded.

PTHrP assay

PTHrP concentrations were measured in conditioned media and in plasma using a two-site immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA), which uses two polyclonal antibodies that are specific for the *N*-terminal-(1–40) and-(60–72) portions of PTHrP and has a calculated sensitivity of 0.3 pmol/liter (28).

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 (unconditioned) used and were considered undetectable if media concentrations were < 0.3 pmol/liter before correction for cell number.

Radiographs and measurement of osteolytic lesion area

Animals were x-rayed in a prone position against the film (22 × 27 cm X-Omat AR, Eastman Kodak Co., Rochester, NY) and exposed with x-rays at 35 KVP for 6 sec using a Cabinet x-ray system-Faxitron Series, Hewlett-Packard Co. (Model 43855A; Faxitron X-Ray Corp., Buffalo Grove, IL). All radiographs were evaluated in blinded fashion. The area of osteolytic bone metastases was calculated using a computerized image analysis system. Video images of radiographs were captured using a frame grabber board (Targa$^+$, Truevision, Inc.) on a PC system. Quantitation of lesion area was performed using image analysis software (Java, Jandal Video analysis, Jandel Scientific, CA).

Bone histology and histomorphometry

Fore- and hindlimb 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 tumor area and osteoclast number per millimeter of tumor/bone interface. Histomorphometic analysis was performed on an OsteoMeasure System (Osteometrics, Atlanta,GA).

Statistical analysis

Results are expressed as the mean ± the SEM. Data were analyzed by repeated measures ANOVA followed by Turkey-Kramer post test. P values of < 0.05 were considered significant.

Results

Ability to support osteoclast formation

Because breast cancers have the ability to induce osteolytic lesions, we determined whether breast cancer cell lines could substitute as an osteoblastic stromal cell to support osteoclast formation. When mouse primary calvarial osteoblasts were cocultured with marrow cells, in the presence of $1\alpha,25(OH)_2D_3$ and PGE_2, for 7 days osteoclasts were formed ($TRAP^+$, MNC, exhibiting CTR and capable of resorbing bone) (Fig. 1A). In contrast, none of the breast cancer lines MDA-MB-231, T47D, MCF-7, and MCF-7 PTHrP 139c were able to support osteoclast formation when cocultured with hematopoietic cells from neonatal mouse spleen or adult mouse

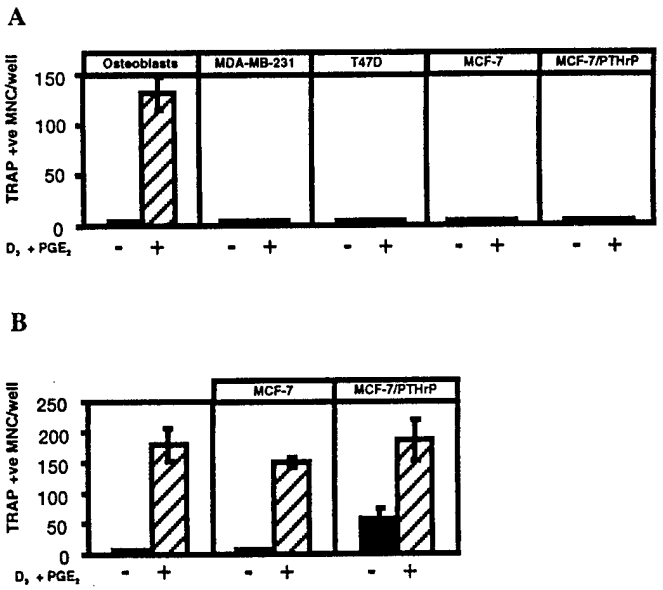

FIG. 1. Influence of breast cancer cells on osteoclast formation. A, Osteoclast formation in cocultures of mouse marrow cells with primary osteoblasts (osteoblast) or breast cancer cell lines in the presence or absence of $1\alpha,25(OH)_2D_3$ (10^{-8} M) and PGE_2 (10^{-7} M). B, Effect of breast cancer cells on osteoclast formation in cocultures of mouse marrow cells with primary osteoblasts. Cocultures were performed in the presence or absence of $1\alpha,25(OH)_2D_3$ and PGE_2 with the addition of MCF-7 cells (MCF-7) or MCF-7 PTHrP 139 cells. After culture for 7 days, $TRAP^+$, multinucleate (> 3) cells were counted. Each coculture was repeated three times in quadruplicate wells and expressed as the means ± SEM.

marrow in the presence of $1\alpha,25(OH)_2\ D_3$ and PGE_2 (Fig. 1A). This indicated that breast cancer cell lines alone were unable to substitute for stromal osteoblasts. No multinucleated osteoclasts were formed when marrow or spleen cells were incubated alone even in the presence of $1\alpha,25(OH)_2D_3$ and PGE_2 as has been previously described (29).

We next addressed whether the presence of breast cancer cells could modify the osteoclast-inductive potential of stromal osteoblastic cells when cocultured with hematopoietic cells from adult mouse marrow (Fig. 1B) or neonatal mouse spleen (data not shown). These models are more akin to the *in vivo* situation, whereby breast cancer cells interact with both osteoblasts and hematopoietic cells. When osteoblastic cells were cultured with hematopoietic cells, osteoclasts were formed in response to treatment with $1\alpha,25(OH)_2\ D_3$ and PGE_2. Similarly, when any of the breast cancer cell lines, MCF-7 (Fig. 1B), MDA-MB-231 or T47D (not shown), were added in equal numbers to the osteoblastic cells (5×10^4 cells/48-well culture plate), osteoclast formation occurred and the resultant osteoclast numbers were similar to that seen with osteoblast and hematopoietic cell cocultures (Fig. 1B). The small discrepency in osteoclast numbers seen between the osteoblastic coculture and the MCF-7 coculture maybe due to the high level of expression of OPG seen in the MCF-7 cells (Fig. 2A), which could diminish osteoclast formation.

Expression of PTHrP by breast cancer cells is known to enhance osteolytic potential of MDA-MB-231 breast cancer cells *in vivo* (2, 30). The effect of a PTHrP-overexpressing cell line (MCF-7 PTHrP 139c) on modulating osteoclast formation in coculture was therefore assessed. In cocultures of osteoblasts and hematopoietic cells in which MCF-7 PTHrP 139c cells (5×10^4 cells/48-well culture plate, numbers equal to osteoblastic cells) were added to the coculture, $TRAP^+$ multinucleated cell formation occurred even in the absence of exogenous osteotropic agents (Fig. 1B). These cells displayed the properties of osteoclasts *i.e.* $TRAP^+$, multinucleated, expressed CTR and could resorb bone. Upon addition of $1\alpha,25(OH)_2\ D_3$ and PGE_2 osteoclast formation was equivalent to that generated by osteoblasts with these treatments (Fig. 1B). The resultant osteoclast formation seen with the PTHrP-overexpressing cells in the absence of any osteotropic agents contrasts markedly to that of the parental MCF-7 cell line.

Because the TNF-related ligand (RANKL) and the receptors (OPG, RANK) have been invoked as pivotal molecules in osteoclast formation (21), we determined the expression of RANKL, OPG, and RANK mRNA in breast cancer cell lines and in primary breast cancers. Total RNA was isolated and reverse transcribed then subjected to PCR for 40 cycles of amplification, which represent saturating, nonquantitative, conditions. As controls, RT-PCR was performed on RNA isolated from a giant cell tumor of bone (GCT), which we have determined to express RANKL, OPG, and RANK. As a negative control, PCRs were performed on RNA, which had not been reversed transcribed and no products were detected under these conditions.

Consistent with the inability of breast cancer cell lines to support osteoclast formation, the lines MDA-MB-231, T47D, MCF-7, and MCF-7 PTHrP 139c did not express RANKL

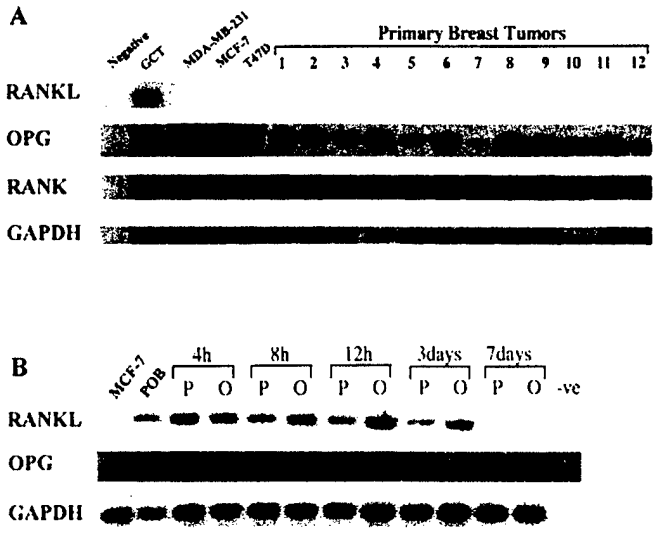

FIG. 2. A, RT-PCR analysis of RANKL, OPG, RANK and GAPDH mRNA in breast cancer cell lines and primary breast cancers. PCR amplification of RANKL mRNA was performed with oligonucleotide primer pairs RANKL-15 and RANKL-16; amplification of OPG mRNA was performed with primers OPG-4 and OPG-5; amplification of RANK mRNA was performed with primers RANK-10 and RANK-11; and amplification of GAPDH mRNA was performed with primers GAPDH-3 and GAPDH-4, as described in the *Materials and Methods*. PCR products were resolved on a 1% (wt/vol) agarose gel, transferred to a nylon membrane and hybridized with an internal oligonucleotide; RANKL-6 (RANKL), OPG-6 (OPG), RANK-15 (RANK) or GAPDH-1 (GAPDH). Lanes correspond to: negative control; giant cell tumor, GCT; MDA-MB-231, MCF-7, T47D breast cancer cell lines and primary breast tumor samples 1–12. This figure is representative of three independent RT-PCRs. B, RT-PCR analysis of RANKL, OPG and GAPDH mRNA in a coculture system of MCF-7 breast cancer cells and murine osteoblastic cells. PCR amplification of RANKL mRNA was performed with oligonucleotide primer pairs RANKL-1 and RANKL-2 specific to murine RANKL mRNA: amplification of murine OPG mRNA was performed with primers OPG-1 and OPG-2; and amplification of murine GAPDH mRNA was performed with primers GAPDH-2 and GAPDH-4, as described. PCR products were resolved on a 1% (wt/vol) agarose gel, transferred to a nylon membrane and hybridized with an internal oligonucleotide; RANKL-11 (RANKL), OPG-3 (OPG) or GAPDH-1 (GAPDH). Lanes correspond to: MCF-7 cells alone, murine primary osteoblastic cells (POB) alone and coculture of murine primary osteoblasts into MCF-7 parental (P) and MCF-7 PTHrP 139c (O) cells for the times indicated. RNA from the 4-h time point which had not been reversed transcribed acted as the control (-ve). This figure is representative of three independent RT-PCRs.

mRNA, whereas the GCT expressed a single product with nucleotide sequence identity to RANKL (Fig. 2A). Additionally, none of the primary breast cancers expressed RANKL mRNA (Fig. 2A). Because RNA from the primary tumors would represent RNA from both cancer cells and from the normal stroma, this implies that both the stroma and breast cancer cells fail to express RANKL. In contrast, the three breast cancer cell lines and all tumors expressed mRNA for OPG and RANK (Fig. 2A); albeit that OPG levels were lower in the T47D cells relative to the MCF-7 and MDA-MB-231 cells as has been reported previously (31). The MCF-7 PTHrP 139c cells showed an equivalent level of OPG mRNA expression to the parental MCF-7 cells (data not shown). Using the GAPDH primers, a product of the predicted size was amplified from each of the tumor and cell line samples,

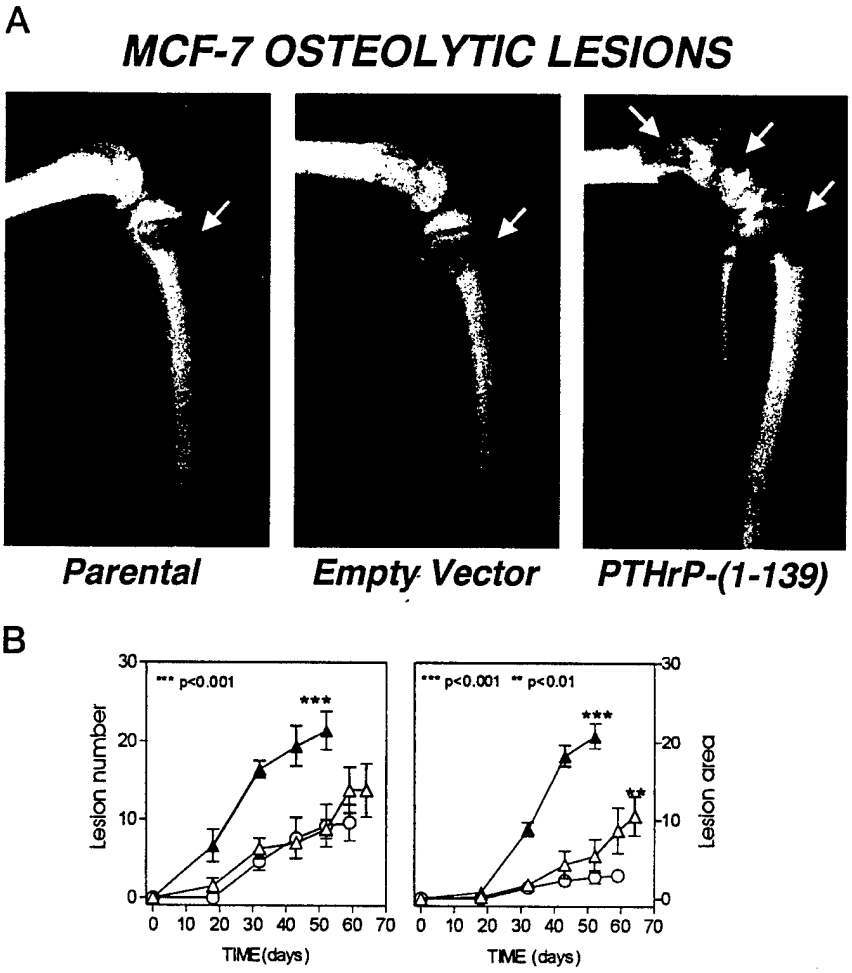

FIG. 3. Representative of hindlimbs from mice bearing parental, MCF-7 pcDNAIneo and MCF-7 overexpressing PTHrP tumors. *Top panel*, Radiographs were taken 40 days after inoculation of tumor cells. Osteolytic lesions are indicated by the *arrows*. The most bone destruction is evident in the mice bearing MCF-7 PTHrP 139c. *Bottom panel*, Quantitation of tumor lesion number and area. Respective tumor cells were inoculated on day 0: mice bearing parental MCF-7 (○), MCF-7 pcDNAIneo (△) and MCF-7 cells overexpressing PTHrP (▲) tumors. Lesion number and area was measured from long bones of fore- and hindlimbs. Values represent the mean ± SEM, n = 5 per group.

which hybridized with the internal GAPDH oligonucleotide, GAPDH-1 (Fig. 2A), thus confirming the integrity of RNA used.

RANKL mRNA is regulated by osteotropic factors in osteoblasts (13, 32) and its expression relative to that of OPG expression appears to dictate the osteoclast-inductive nature of cells (32). Thus, we examined the effect of local and systemic factors such as IL-11, PTHrP, transforming growth factor β (TGFβ) and 1α,25(OH)$_2$ D$_3$ to stimulate the expression of RANKL in the MDA-MB-231, T47D, and MCF-7 cell lines. However, none of these agents permitted the expression of RANKL in any of these cell lines (data not shown), suggesting that the *RANKL* gene in breast cancers is maintained in a transcriptionally inactive state. Such a result is in accordance with the inability of breast cancer cell lines to act in a manner equivalent to osteoblastic stromal cells for differentiation and induction of osteoclasts from hematopoietic cells.

PTHrP expressed by breast cancers modulates osteoblast RANKL production

We have previously established that osteoblastic RANKL mRNA levels can be enhanced by PTH/PTHrP (32). To determine if PTHrP produced by breast cancer cells invoked changes in RANKL mRNA similar to those we observed in response to treatment of primary osteoblasts, murine RANKL and OPG mRNA levels were assessed in cocultures of human breast cell lines and primary murine osteoblasts (Fig. 2B). MCF-7 parental and the PTHrP overexpressing cell line, MCF-7 PTHrP 139c, were cultured with primary mouse osteoblasts (osteoblasts at 1×10^6 cells and MCF-7 at 5×10^5 cells per 10 mm Petri dish), and murine RANKL and OPG levels were determined during 7 days of coculture. Mouse RANKL mRNA was induced 2.4 ± 0.2-fold following 8 h of coculture with MCF-7 PTHrP 139c cells and remained elevated for up to 72 h. Levels of murine RANKL mRNA were unaltered in cocultures comprising MCF-7 cells for up to 3 days, then in both MCF-7 and MCF-7 PTHrP 139 cocultures murine RANKL mRNA levels decreased by day 7 (Fig. 2B). Furthermore, murine OPG mRNA expressed by osteoblastic cells was reduced 6.1 ± 0.5-fold at 8 h when MCF-7 cells overexpressing PTHrP were cocultured with murine osteoblasts compared with parental MCF-7 cells.

In vivo experiments

Given that the MCF-7 cells overexpressing PTHrP enhanced osteoclast formation in murine *in vitro* cocultures, we sought to establish the role of PTHrP overexpression by the MCF-7 cell line *in vivo* by intracardiac injection in the nude mouse model. Such a model has demonstrated that MDA-

MB-231 cells avidly metastasize to bone and induce osteolysis (30).

Mice inoculated with the MCF-7 PTHrP 139c developed large bone metastases with osteolysis being evident earlier and to a greater extent than that seen with mice harbouring either the parental cells or cells stably transfected with the vector control only (Fig. 3). When quantitated by computerized image analysis of radiographs, the differences in lesion area and number were statistically significant (Fig. 3). The *in vitro* growth rates of these MCF-7 cells showed no significant difference between the MCF-7 PTHrP-139 and the empty vector or parental cells (data not shown) therefore the difference in tumor size is not attributable to the growth rate of the various MCF-7 cells. Histomorphometric analysis of bone (Fig. 4, A and B) indicate that osteoclast number per mm of tumor-bone interface was markedly greater, as was tumor area, in mice bearing MCF-7 PTHrP-139 tumors compared with the control parental and empty vector groups. Significant hypercalcemia was evident in mice bearing the MCF-7 PTHrP-139 cells, whereas mice bearing the MCF-7 pcDNAIneo (empty vector) or parental cells, remained normocalcemic (Fig. 4C). Concomitant with this hypercalcemia, plasma PTHrP concentrations were significantly greater at the time mice were killed (Fig. 4D) in the MCF-7 PTHrP-139c bearing mice. There was no significant difference in body weight between mice bearing tumors of MCF-7 PTHrP 139c, empty vector or parental cells (not shown).

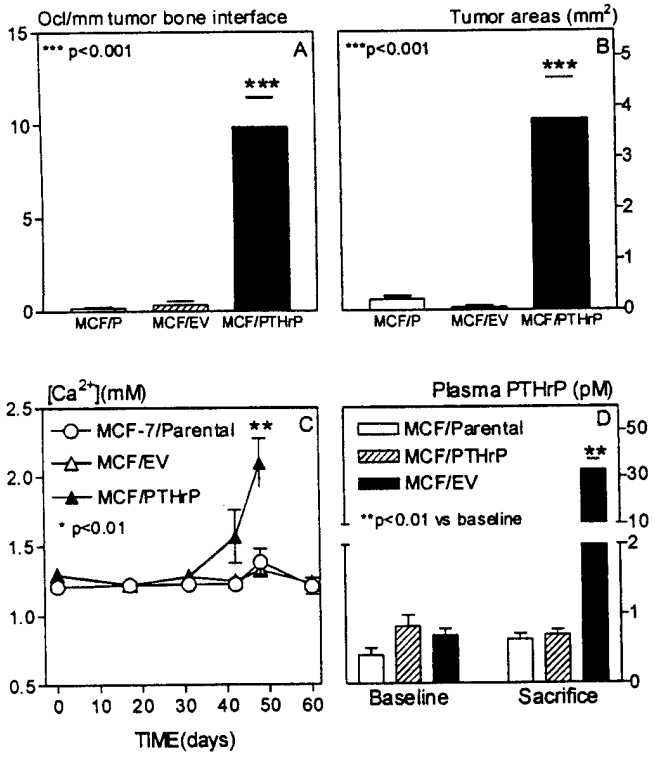

FIG. 4. *In vivo* metastasis. A, Osteoclast number per mm of tumor-bone interface and B, tumor area as assessed by bone histomorphometry of hind limbs from mice bearing parental MCF-7 (*open*), MCF-7 empty vector (*hatched*) and PTHrP overexpressing (*closed*) tumors. C, Whole blood ionized calcium concentrations in mice bearing parental MCF-7 (○), MCF-7 pcDNAIneo (△) and MCF-7 cells overexpressing PTHrP (▲) tumors. Calcium concentrations were significantly higher in mice bearing the MCF-7 PTHrP overexpressing tumors compared with the other groups. Values represent the mean ± SEM, n = 5 per group. D, Plasma PTHrP concentrations in mice bearing parental MCF-7 (*open*), MCF-7 pcDNAIneo (*hatched*) and MCF-7 PTHrP 139c (*solid*) tumors. Plasma PTHrP concentrations at the mice were killed were significantly higher than respective concentrations before tumor inoculation (baseline) in mice bearing MCF-7 PTHrP 139c tumors. Values represent the mean ± SEM, n = 5 per group.

Discussion

It is well established that breast cancers have the capability to establish and grow as metastases in bone, however the mechanism underlying their ability to induce osteolysis remains unresolved. Although some authors have suggested that breast cancer cells alone have the capacity to degrade the bone matrix, lesions of bone or dentine slices attributable to breast cancer cells observed *in vitro* are not of the magnitude of those resulting from osteoclast-mediated bone destruction (1). Our present series of experiments provide unequivocal evidence that several breast cancer cells were unable to act as surrogate osteoblasts to support osteoclast formation *in vitro*. Moreover, breast cancer cell lines such as T47D, MCF-7, and MDA-MB-231 do not express the TNF-related ligand, RANKL, which is required along with M-CSF for the differentiation of hematopoietic cells into osteoclasts. Further RANKL mRNA expression could not be induced in response to treatment with any osteotropic agents known to enhance osteoclast formation, and no expression was detected in a series of surgically obtained primary breast cancers.

Several studies have implicated PTHrP as a crucial factor in the process of breast cancer metastases in bone (2, 30). This study extends these observations to demonstrate that PTHrP produced by breast cancer cells is sufficient to stimulate osteoclast formation in marrow cultures with osteoblastic cells without the requirement for exogenous osteotropic agents such as 1,25α $(OH)_2$ D_3, PGE_2, or IL-11. In such cocultures we show the capacity of breast cancer cell-derived PTHrP to induce osteoblastic RANKL mRNA levels and reduce osteoblastic OPG mRNA levels, the net effect of which is anticipated to enhance osteoclast formation. Such a finding is concordant with the osteoclast induction seen when breast cancer cells overexpressing PTHrP were added to cocultures and to previous *in vitro* experiments where exogenous PTH/PTHrP similarly modulate RANKL and OPG mRNA levels (32).

Finally, and most significantly, we extend these *in vitro* findings to an *in vivo* model of metastasis using the well established nude mouse model whereby cancer cells were administered via intracardiac injection. Whilst parental MCF-7 cells had a low prevalence for metastasis in bone, when PTHrP was overexpressed the cells avidly metastasized to bone and induced osteolysis with accompanying hypercalcemia. The MCF-7 cells have not been shown previously to be capable of metastatic growth in the nude mouse.

Combining the *in vitro* and *in vivo* data, a possible model for the severe osteolysis induced by breast cancers is proposed in Fig. 5. This model extrapolates from our previous findings (2) and results presented herein. As a consequence of breast cancer cells establishing in the bone microenviron-

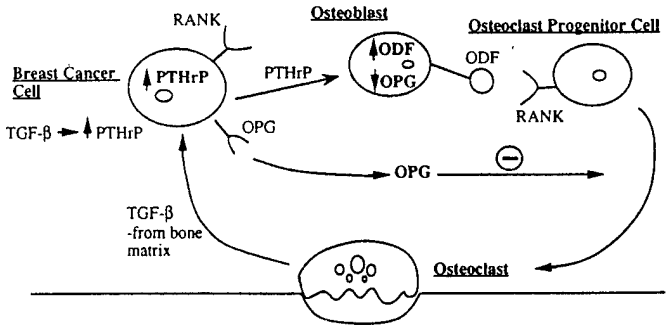

FIG. 5. Potential effects of PTHrP on osteoclast formation. Breast cancer cells express OPG, RANK and PTHrP. Secreted PTHrP leads to an enhancement of osteoblastic RANKL mRNA and reduction in osteoblastic OPG mRNA. RANKL engages its cognate receptor RANK on cells at the macrophage/monocyte lineage permitting differentiation of these cells into functional osteoclasts. Finally, as a result of bone resorption, TGFβ, a potent enhancer of PTHrP production, is released from the bone matrix: TGFβ may also act on the osteoblast to enhance PTHrP production from osteoblast. Such a cycle would potentiate the osteolytic potential of tumors once established in bone.

ment, PTHrP secreted from these cells can act in a paracrine/juxtacrine manner on osteoblastic cells, increasing RANKL expression and limiting OPG expression. This favors the formation and the survival of osteoclasts because RANKL has also been demonstrated to limit osteoclast apoptosis (14). Enhancement of osteoclast numbers and their activity results in pronounced osteolysis with the subsequent release of bone-derived growth factors such as TGFβ. TGFβ is a potent stimulator of PTHrP production acting both transcriptionally and posttranscriptionally via mRNA stabilization (33, 34). Recently, TGFβ has been demonstrated to decrease RANKL mRNA and enhance OPG mRNA levels in osteoblasts (35). Such a mechanism may well account for the local control of bone formation and resorption. However, when breast cancer cells have established in the bone environment, differential roles of bone-derived growth factors, including TGFβ, can emerge. Thus for example, they could modify the production of cytokines and growth factors by breast cancer cells and osteoblastic cells in ways that could influence RANKL, OPG and the signaling receptor RANK.

This work provides conclusive evidence that breast cancers are incapable of stimulating osteoclast formation as a result of a direct interaction with hematopoietic cells, but facilitate their lytic potential via the osteoblast. The demonstration that breast cancer cell-derived PTHrP can modulate osteolysis, coupled with the knowledge that OPG can limit osteoclast formation, provides two unique secreted targets to address for therapy to limit osteolysis as a result of breast cancer metastasis in bone.

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