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This proposal aims to study the role of PTHrP in breast cancer-mediated hypercalcemia and osteolysis. Results thus far demonstrate that 1 of 4 breast cancer cell lines screened (MDA-MB-231) secretes PTHrP *in vitro* and this was not related to the presence of the estrogen receptor. *In vivo* data demonstrate that tumor-mediated osteolysis is associated with osteoclastic bone resorption at sites of tumor involvement and that PTHrP causes hypercalcemia when produced at sites distant from bone and locally by tumor in bone. Localized osteolysis mediated by PTHrP may occur without hypercalcemia. These data suggest that different tumor syndromes may be associated with PTHrP production and depend on the tumor location and degree of PTHrP expression. Additional *In vivo* data demonstrate that PTHrP overexpression in a breast cancer cell line is associated with an increased number of osteolytic metastases, hypercalcemia without an increase in plasma PTHrP concentration, and decreased survival. These data support the clinical findings that PTHrP, as a bone resorbing factor, may contribute to the ability of breast cancer to grow in bone and have important implications in the prognosis of women with newly diagnosed breast cancer as well as in the treatment of breast cancer metastatic to bone.

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Theresa A Hunn, 10/26/95

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

NATURE OF THE PROBLEM AND BACKGROUND OF PREVIOUS WORK

Breast Cancer, Hypercalcemia and Osteolysis

Breast cancer is associated with significant morbidity in the skeleton. Specifically, breast cancer can involve bone through both metastatic and humoral mechanisms. Metastases to bone are more commonly osteolytic than osteoblastic and are responsible for the complications of bone pain, pathologic fracture, hypercalcemia and nerve compression syndromes that many breast cancer patients suffer from (1). Eighty-four per cent of patients dying of breast cancer have bone metastases (2).

Hypercalcemia is commonly associated with breast cancer, occurring in up to 40% of afflicted women during the course of their disease (2,3). Skeletal destruction by metastatic tumor has been felt to be the major mechanism responsible for hypercalcemia (3). Increased osteoclastic bone resorption in areas surrounding breast cancer metastasis has been documented histologically (4,5) suggesting that factors secreted by breast cancer cells can locally activate osteoclasts. Recent evidence, however, suggests that osteolytic bone metastasis may not be the only mechanism responsible for breast cancer hypercalcemia and that humoral mechanisms may contribute in as much as 30-60% of the cases (6-8). One investigator found that 15% of 147 hypercalcemic breast cancer patients had no bone metastases (9).

PTHrP and Breast Cancer

Parathyroid hormone-related protein (PTHrP) is a major mediator of humoral hypercalcemia of malignancy, due to its PTH-like actions. This protein was purified in 1987 from human lung cancer (10), breast cancer (11) and renal cell carcinoma (12) simultaneously by several independent groups. Cloning and expression followed shortly thereafter (13).

PTHrP has since been extensively studied and found to have many similarities to PTH. It has 70% homology to the first 13 amino acids of the N-terminal portion of PTH (13), binds to PTH receptors (14) and shares similar biologic activity to PTH (15). Specifically, it stimulates adenylate cyclase in renal and bone systems (11,12,15-17), increases renal tubular reabsorption of calcium and osteoclastic bone resorption (16,17), decreases renal phosphate uptake (15,16,18) and stimulates 1α-hydroxylase (15). PTHrP has been found in a variety of tumor types as well as normal tissue (19-22). The widespread expression of PTHrP in normal as well as malignant tissue was the first evidence that the hormone has a role in normal physiology. In addition to the PTH-like effects, PTHrP has many non-PTH-like properties (23), some of which include regulation of placental calcium transport (22), possible establishment of bone metastasis in breast cancer (24,25), and autocrine regulation of the growth of some tumors (26). The regulation of PTHrP is poorly understood, but factors such as prolactin (27), glucocorticoids, $1,25(OH)_2D_3$ (28), epidermal growth factor (28), TGFα (29), TGFβ (30), estrogen (31-34) and stretch (35) have been shown to regulate gene expression and extracellular calcium concentration has been shown to control the production of PTHrP in vitro in Leydig tumor cells (36).

It is now clear that PTHrP is a significant factor in mediating hypercalcemia in breast cancer (37). One of the 3 tumors from which PTHrP was originally purified was a breast cancer from a patient with humoral hypercalcemia of malignancy (11). PTHrP was detected by immunohistochemical staining in 60% of 102 invasive breast tumors removed from normocalcemic women, but not in normal breast tissue (24). By immunohistochemistry (25) and in situ hybridization (38), it was detected in 12 of 13 breast cancer metastases in bone leading to the speculation that production of PTHrP as a bone-resorbing agent may contribute to the ability of breast cancers to grow as bone metastasis. Along these lines, Bundred and colleagues found positive immunohistochemical staining for PTHrP in 56% of 155 primary breast tumors from normocalcemic women and PTHrP positivity was related to the development of bone metastases (39). Additionally, 65-92% of hypercalcemic breast cancer patients (with and without bone metastasis) had detectable plasma PTHrP concentrations by radioimmunoassay (RIA) similar to those documented in patients with humoral hypercalcemia of malignancy due to non-breast tumors (40,41).

PTHrP in Nonmalignant Breast Disease

In addition to its role in malignancy, PTHrP is important in the normal physiology of breast (42). It is expressed in lactating mammary tissue (43) and secreted into milk at concentrations 10,000-100,000 times greater than plasma concentrations of humans with malignancy-associated hypercalcemia (44-48). Suckling increases PTHrP gene expression and this appears to be mediated through prolactin (49). Estrogen has been shown to increase PTHrP expression in uterine tissue and *in vitro* studies suggest that there may be estrogen response elements present in the PTHrP gene (50-53). Increased plasma PTHrP concentrations have been described in at least 2 patients with the rare syndrome of lactational hypercalcemia (54-56). Animal studies have demonstrated a PTHrP gradient across the mammary gland in lactating goats (48) indicating that PTHrP may gain access to the maternal circulation during lactation. In support of this, a recent clinical study has shown detectable plasma PTHrP concentrations in 63% of breast-feeding mothers while similar measurements in bottle-feeding control mothers were undetectable (57). Thus, PTHrP may be responsible for mobilizing calcium from maternal bone for use in milk production. Indeed, it may be the implicating factor in lactation-associated bone loss (58).

PTHrP as a Growth Regulator

PTHrP has recently been shown to be produced in relatively low concentrations in breast myoepithelial cells (59). Interestingly, a transgenic mouse model, in which PTHrP is over expressed in skin and breast myoepithelial cells through the use of a human keratin promoter, has demonstrated breast hypoplasia. Specifically, female transgenics had a severe reduction in the number of albeit normal terminal ducts and acini in the breast suggesting that PTHrP may play a role in regulating ductular proliferation and/or differentiation during mammogenesis (60). These mice also had failure of normal hair follicle development indicating a similar role for PTHrP in the skin.

Along those lines, disruption of PTHrP expression in a normal keratinocyte cell line, using antisense technology, results in enhanced growth of the cells in culture (61). *In vivo*, homozygote mice for the PTHrP null mutation are born with a multitude of skeletal abnormalities, including defects in the bone growth plate (62). These findings, along with those of the above described transgenic mice, suggest that either over- or under- expression of PTHrP in normal cells result in abnormalities of growth and possibly differentiation.

In malignant cells, PTHrP has been shown to act as an autocrine growth factor in a

renal cell carcinoma cell line (26) and more recently, in a squamous cell carcinoma line (63). There are no reported studies on the role of PTHrP as an autocrine growth factor in breast cancer.

Regulation of PTHrP by Other Tumor-associated and Bone-derived Growth Factors

Other tumor-associated growth factors as well as bone-derived growth factors may be important regulators of PTHrP expression in both malignant and non-malignant tissue. Epidermal growth factor has been shown to increase PTHrP expression in a keratinocyte cell (64) line while TGF-α, a breast cancer tumor product (65), enhances PTHrP expression in a human squamous cell carcinoma of the lung (29). Moreover, other tumor-associated factors may modulate the end organ effects of PTHrP. The principal investigator has shown that TGF-α enhances the hypercalcemic effects of PTHrP in an animal model of malignancy-associated hypercalcemia (66) and others have demonstrated that TGF-α can modulate the renal and bone effects of PTHrP (67,68). Additionally, TGF-β, which is present in high concentrations in the bone microenvironment, has been shown to enhance secretion of and stabilize the message for PTHrP in a renal cell carcinoma (30) as well as in an epidermal squamous cell carcinoma (69).

Implications of PTHrP Status in Breast Cancer

These findings have important implications for the ability of breast cancer to affect the skeleton. First, breast cancers expressing PTHrP in addition to other tumor-associated factors, such as TGF-α (65), may be more likely to affect the skeleton through humoral and osteolytic mechanisms if the co-expressed factor enhances PTHrP expression in the primary tumor. Second, if estrogen can regulate PTHrP expression in breast cancer cells as it can in other tissues, estrogen receptor positive tumors may preferentially express PTHrP. Finally, growth of breast cancer cells in bone may be enhanced if the tumor cells express PTHrP. TGF-β, as well as other bone derived growth factors, are present in high concentration in the bone microenvironment (70) and are released from bone during the process of osteoclastic bone resorption (71). PTHrP expression in breast cancer cells lodged in bone is likely to be increased in the presence of TGF-β. In this scenario, osteoclastic bone resorption is increased further causing release of more TGF-β and other growth factors into the bone microenvironment leading to further enhancement of PTHrP expression in the breast cancer cells. If PTHrP acts as an autocrine growth factor in breast cancer cells, as it does in some tumor models, then tumor growth would be enhanced as well. The clinical findings of an increased incidence of PTHrP expression in bone compared with other sites by Powell and colleagues (25,38) 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.

If PTHrP expression in the primary breast tumor indicates a propensity to metastasize to bone due to its potent bone resorbing capability, early treatment with inhibitors of bone resorption is 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. 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 (72-74). Bisphosphonates have also been shown to decrease the number of bone metastases in animal models (75,76), but it is unclear whether or not these tumors express PTHrP. However, since the safety of long term bisphosphonate use has not been determined and bone mineralization defects can occur with high doses of these drugs, it would be of benefit, as well as cost effective, to identify which patients are at risk to develop bone metastases and treat only those rather than treat all women with breast cancer. The clinical evidence thus far

supports PTHrP as a marker to identify such women, but better animal models are needed to clarify this role.

Knowledge of PTHrP status may also have significant therapeutic implications in treating breast cancer-associated hypercalcemia. Although hypercalcemia in breast cancer is often associated with bone metastases, it is clear that humoral mechanisms may contribute in as much as 60% of the cases. Traditionally, treatment has been directed toward inhibiting bone resorption and this is often effective. However, it has now become evident that bisphosphonate therapy is less effective in patients with higher plasma concentrations of PTHrP and without radiological evidence of bone metastases (77,78). Thus, inhibition of bone resorption is effective when the major mechanism for hypercalcemia is increased bone resorption. Since PTHrP causes hypercalcemia by both increasing osteoclastic bone resorption and increasing renal tubular reabsorption of calcium, drugs that inhibit bone resorption alone may not normalize the calcium concentration if the plasma PTHrP concentration is high enough to add a significant renal component to the hypercalcemia. Drugs directed against either the actions of PTHrP or the secretion of PTHrP may therefore be more beneficial in the bisphosphonate resistant situation. Unfortunately, no such drugs are available at the current time but the need for them is obvious. A potentially useful therapy may prove to be the use of monoclonal antibodies against PTHrP. Sato has recently described successful use of an anti-PTHrP-(1-34) monoclonal murine antibody in an animal model of humoral hypercalcemia that ameliorated hypercalcemia and prolonged survival time in severely ill animals (79).

In Vivo Models of Hypercalcemia and Osteolysis

These observations demonstrate the need for further study of the role of PTHrP in malignant and nonmalignant breast disease. The only research done to date on PTHrP expression in human breast cancer and its potential role in humoral hypercalcemia and the development of osteolytic bone metastases have involved the small clinical studies described above (24,25,39-41). Despite numerous animal models of human breast cancer (80) that have been described to date, no human breast tumor line has been studied *in vivo* for PTHrP expression and its relationship to the development of osteolytic bone metastases and humoral hypercalcemia. Most animal models of breast cancer have been used to evaluate the effect of various factors (81-83) on breast cancer growth. Only one spontaneous rat mammary tumor (Walker 256 carcinosarcoma) has been shown to cause humoral hypercalcemia in rats (84), produce PTHrP (85) and cause osteolytic bone metastases (75). Given the accumulating evidence documenting a humoral mechanism for hypercalcemia in breast cancer, the established role of PTHrP in humoral hypercalcemia of malignancy, the presence of PTHrP in malignant as well as lactating breast tissue and the presence of PTHrP in established breast cancer metastases to bone, it is evident that established models of human breast cancer should be evaluated for PTHrP expression and its relationship to the skeleton. Using animal models will be beneficial in defining this aspect of the pathophysiology of breast cancer and this will in turn have important prognostic and therapeutic implications.

Historically, it has been difficult to produce bone metastases in animal models of malignancy. Tumors inoculated subcutaneously or intramuscularly do not metastasize in nude mice and tumors inoculated into the tail vein usually produce only lung metastases. Yoneda has developed an animal model of human breast cancer cell metastasis to bone (76,86) which is based on a model originally described by Arguello (91). In this model, MDA-MB-231 breast cancer cells injected into the left ventricle of nude mice reliably produce osteolytic lesions that are evident radiologically as well as histologically. This model has been used to show that the bisphosphonate, risedronate, decreased osteolytic lesions when given simultaneously with tumor cells and inhibited both an increase in new bone metastases and progression of each metastatic focus when give to animals with pre-existing osteolytic lesions (76).

PURPOSE OF PRESENT WORK

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 of increasing 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 its potent bone resorbing capacity, PTHrP expression in the primary tumor may aid in establishment of the bone metastases that are so 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 is to define the role of PTHrP in the pathophysiology of breast cancer using animal models of breast cancer-mediated humoral hypercalcemia and osteolytic bone metastases. Information gained from these studies will have important prognostic and therapeutic implications.

METHODS OF APPROACH

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 objectives are 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.

BODY

METHODS

Cell culture

Cell lines were cultured in respective media, grown to confluence in T75 flasks, washed with serum-free medium and incubated for 24 hours in serum-free medium. Cell number was counted following trypsinization and conditioned medium was collected with protease inhibitors and stored at -70°C until assayed for PTHrP. Detection of PTHrP was performed using a 2-site immunoradiometric assay (IRMA) commercially available from Nichols Institute (San Capistrano, CA) that is further described below. PTHrP concentrations in conditioned media were corrected for cell number. MDA-MB-231, MCF-7, ZR-75-30,T-47D, and CHO cells were obtained from the American Type Culture Collection (Rockville, MD). CHO cells expressing full-length PTHrP-(1-141) in the sense [CHO/PTHrP-(1-141)] or antisense [CHO/PTHrP-(1-141)-AS] orientation were constructed by the Principal Investigator as previously described (90). RWGT2 is a human squamous cell carcinoma of the lung that was derived from bone metastases, secretes high quantities of PTHrP and was established in the Principal Investigator's laboratory (66).

MDA-MB-231, RWGT2 and CHO cells were grown in DMEM with 10% fetal calf serum (FCS) and 1% non-essential amino acids. ZR-75-30 cells were grown in RPMI 1640 with 1mM sodium pyruvate and 10% FCS. T-47D cells were grown in RPMI 1640 with 0.2 IU bovine insulin/ml and 10% FCS. MCF-7 cells were grown in EMEM with non-essential amino acids and sodium pyruvate and 10% FCS.

PTHrP measurement

Immunoreactive PTHrP was measured in conditioned media and plasma using a 2 site IRMA kit available from Nichols Institutes. This assay utilizes 2 different polyclonal antibodies to PTHrP (1-40 and 60-72) and will detect only peptides that bridge these antibodies. All samples, conditioned media and plasma were collected in protease inhibitors and stored at -70°C until assay, as PTHrP is very labile. The assay is performed on aliquots of fifty to two hundred microliters. PTHrP concentrations were determined from a standard curve using a computerized RIA program. The sensitivity of the assay is 0.3 pmol/L and specificity to human PTHrP-(1-86) is 100% and 0% with PTH. Intra-assay variation is between 2.9-9.5% and inter-assay variation between 5.3-5.6%.

Transfections and construction of stable clones

The expression vector, pCMVPL1, in which the cDNA for full-length human PTHrP-(1-141) sense and antisense is inserted, is illustrated in figure 1. Transfection of MDA-231 breast cancer cells with pCMVPL1PTHrP(sense) or pCMVPL1PTHrP-AS(antisense) was performed by calcium phosphate precipitation with 10% pRSVneo selection marker as described previously (87). Transient expression was tested on media conditioned for 36 hours using the PTHrP 2-site IRMA (Nichols Institute). G418 at 400µg/ml was added to cells in culture to select stable clones. Cloning was performed by limiting dilution and screened by the above PTHrP assay. The MDA-231/PTHrP-sense clone producing the highest amount of PTHrP (MDA/PTHrP-1) and the MDA-231/PTHrP-AS clone producing the lowest amount of PTHrP was selected for the *in vivo* metastasis studies.

In vivo experiments

1. Technical aspects

a. Humoral hypercalcemia studies: In these experiments, tumor cells were inoculated intramuscularly into the right thigh and standard parameters of calcium homeostasis were monitored. In this model of local tumor development, tumors do not metastasize to bone. Thus the systemic effects of tumor-produced PTHrP on the development of hypercalcemia can be

studied.

Experimental groups included MDA-MB-231, a human breast cancer cell line which secretes low, but detectable amounts of PTHrP, and RWGT2, a human squamous cell carcinoma of the lung that secretes high concentrations of PTHrP and caused humoral hypercalcemia in the patient from which it was established. Each group contained 5 female athymic nude mice, 4-6 weeks old. Mice were inoculated with 1 x 10^7 cells, suspended in 0.1ml PBS, intramuscularly into the thigh. In each group, whole blood (obtained by retro-orbital puncture) was obtained for ionized calcium (Ca^{++}) determination on day 0, 7, 10 and every third day thereafter until sacrifice. Ca^{++} was measured using the Ciba Corning 634 ISE Ca^{++}/pH analyzer as described below (87). Body weight and tumor volume was measured on these days as well. Tumor volume was calculated using the formula for an ovoid: Volume = $4/3\pi$ x L/2 x $(W/2)^2$ (L is longest axis and W is mean axis width). Tumor diameters are measured with calipers. Mice were sacrificed when moderately hypercalcemic (Ca^{++} >2.00mmol/L) or ill-appearing and whole blood was collected in protease inhibitors for PTHrP determination. Vertebral and calvarial bones were harvested and saved in formalin for histologic and histomorphometric analysis.

b. Bone metastasis studies: MDA-MB-231 breast cancer cells injected into the left ventricle of nude mice reliably produce osteolytic lesions that are evident radiologically as well as histologically. Figure 2 pictorial illustrates the technique. In this model, nude mice are inoculated into the left cardiac ventricle with a tumor cell suspension. In 3 weeks, osteolytic lesions are evident on radiographs (illustrated in top right panel of figure 2; arrows pointing to osteolytic lesions). Histologic sections of these lesions reveal tumor adjacent to osteoclasts resorbing bone (illustrated in lower right panel of figure 2).

Tumor cells are grown to confluence, trypsinized, washed twice with PBS and resuspended with PBS to a final concentration of 10^5 cells/100µl immediately prior to inoculation. Animals are deeply anesthetized with pentobarbital (0.05 mg/g) and positioned ventral side up. The left cardiac ventricle is punctured through a percutaneous approach 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 indicates correct position in the left cardiac ventricle. Tumor cells are then inoculated slowly over 1 minute.

Three weeks following tumor inoculation, whole-body radiographs were obtained and compared to baseline radiographs obtained prior to tumor inoculation to follow the progression of osteolytic lesions. Ca^{++} and body weight were measured weekly for two weeks post tumor inoculation and then every three days thereafter for the remainder of the experiment. Animals were sacrificed when paraplegic or severely cachectic. At the time of sacrifice, blood was collected for Ca^{++} as well as PTHrP measurement and all bones and soft tissues were harvested in formalin for histologic analysis. Autopsy was performed on all mice and those with tumor adjacent to the heart were excluded from analysis as this indicates that part of the initial tumor inoculum did not properly enter the left cardiac ventricle.

2. Experimental design:

a. Experiment 1: Ability of PTHrP-producing breast cancer (MDA-MB-231) to cause humoral hypercalcemia and osteolytic metastasis. Initial screening of breast cancer cell lines for PTHrP production indicated that MDA-MB-231 produced low, but significantly detectable amounts of PTHrP (figure 3) when compared with CHO-K1 (non-PTHrP-producing) or RWGT2 (high PTHrP-producing). Thus, MDA-MB-231 was compared with RWGT2 for its capacity to cause humoral hypercalcemia and bone metastases using the above experimental approaches. Four groups of mice (n=5 for each) were studied and included: *Local tumor groups (1 & 2):* MDA-MB-231 or RWGT2 (10^7 cells) inoculated intramuscularly into the right thigh; *Metastatic tumor groups (3 & 4):* MDA-MB-231 or RWGT2 (10^5 cells) inoculated into the left cardiac ventricle.

Prior to tumor inoculation, blood was obtained from all mice for measurement of Ca^{++} and PTHrP concentration and baseline whole-body radiographs were obtained. Respective tumor cells were inoculated at respective sites (intramuscular or intracardiac) on day 0. Ca^{++}

and PTHrP concentrations were followed weekly and whole-body radiographs were taken at three weeks post tumor inoculation and at sacrifice. Mice were sacrificed when paraplegic, hypercalcemic or cachectic. Calvarial, vertebral and long bones as well as soft tissues were harvested in formalin at sacrifice and Ca^{++} and PTHrP concentrations were determined from a terminal bleed.

Experiment 2: The effect of PTHrP-overexpression by MDA-MB-231 cells on bone metastases. Since MDA-MB-231 cells produced low amounts of PTHrP and, when inoculated into the left cardiac ventricle of nude mice, osteolytic metastases, MDA-MB-231 cells that overexpress PTHrP (MDA/PTHrP-1) or underexpress PTHrP (MDA/PTHrP-AS) were compared with parental MDA-MB-231 cells in the nude mouse model of bone metastases. Cell lines (MDA/PTHrP-1 and MDA/PTHrP-AS) were constructed as described earlier. Experimental groups included MDA/PTHrP-1, MDA/PTHrP-AS and MDA-MB-231 (nontransfected, parental cells) (n=10 mice/group). Respective tumor cells (10^5) were inoculated into the left cardiac ventricle on day 0. Ca^{++}, body weight, PTHrP measurements, radiography, scoring of bone metastases and bone histology were performed as described in experiment 1. The number of osteolytic lesions were counted at 3 weeks post tumor inoculation. An identical experiment was performed with the exception that the endpoint was survival.

Whole blood ionized calcium measurement

Whole blood ionized calcium concentrations were measured using a Ciba Corning 634 ISE Ca^{++}/pH analyzer, which uses a sample volume of 35 μl. The calcium values are adjusted using the internal algorithm of the instrument pH of 7.4. Samples were run in duplicate and the mean value recorded. The analyzer were calibrated before and after running each group of samples using Ciba Corning 634 Slope Standard (Ca 2.50/pH 6.84). Many published studies have utilized this method (66,87,95).

Bone histology

Calvarial, long and vertebral bones removed from mice were fixed in 10% buffered formalin. The posterior halves of calvarial bones were decalcified in 14% EDTA and embedded in paraffin after processing. Sections were cut using a standard microtome and placed on silane or poly-L-lysin-coated glass slides. Several consecutive sections will be cut at regular levels throughout the specimens. Sections for histomorphometry were stained using hematoxylin and eosin, orange G and phloxine.

Immunohistochemistry

Tumor sections were processed and cut as described above, with the exception that samples were not decalcified in EDTA. Sections were then stained using a standard avidin-biotin immunoperoxidase technique (96). Sections used for immunocytochemistry were de-waxed in xylene for 5-10 minutes, and rehydrated through graded ethanols. Sections were then incubated with a protein retrieval citrate buffer (0.05M) and autoclaved for 10 minutes on the liquid cycle, cooled and washed in water followed by TBS. To block endogenous peroxidase, sections were incubated for 30 minutes in hydrogen peroxide (0.66 %) in 80% methanol, TBS, followed by 5 minute wash in TBS. Nonspecific staining was blocked by incubating the sections for I hour in 20% normal swine serum in TBS. Primary antibody (rabbit anti-human PTHrP-(1-34) obtained from T.J. Martin, Melbourne, Australia) or control was diluted 1:300 in TBS and sections were incubated in this for 30 minutes followed by a wash with TBS containing 5% fetal calf serum. Sections were then incubated with the secondary antibody (biotinylated swine anti-rabbit immunoglobulin, DAKO) at 1:400 dilution for 30 minutes, washed in TBS with 5%FCS and incubated in avidin-biotin complex for 30 minutes. Antibody binding was visualized using 0.5% diaminobenzidine in tris-HCl with 0.03% hydrogen peroxide as the peroxidase substrate. Sections were counterstained with Harris haematoxylin.

Scoring of bone metastases

The number of osteolytic bone metastases was determined on radiographs as described by Nakai (86). At the end of the experiments, animals were anesthetized deeply with pentobarbital, laid down in a prone position against the films (22 x 27 cm X-O mart AR, Kodak) and exposed with an x-ray at 35 KVP for 6 seconds using a Faxitron Radiographic Inspection Unit (Model 8050-020, Field Emission Corporation, Inc.). Films were developed using a RP X-O Mart processor (Model M6B, Kodak). All of the radiographs of the bones in nude mice were evaluated extensively and carefully by 3 different individuals in a blinded fashion. From the radiographs, osteolytic metastatic foci as small as 1 mm in diameter, which are recognized as demarcated radiolucent lesions in the bones, were enumerated.

Statistics

Unless otherwise indicated, values are reported as the mean ± standard error of the mean. Statistical significance was determined using analysis of variance followed by Tukey's post test with Graphpad Instat software on an IBM compatible computer.

RESULTS

Initial screening of breast cancer cell lines for PTHrP production *in vitro* by measuring immunoreactive PTHrP in media conditioned by respective cell lines is illustrated in table 1. Of the cell lines tested, only MDA-MB-231 cells secreted significant amounts of full-length PTHrP. However, the amount of PTHrP produced by MDA-MB-231 was significantly less than that produced by RWGT2, a human squamous cell carcinoma that causes humoral hypercalcemia. There was no detectable PTHrP in media conditioned by ZR-75-30, T-47D or MCF-7 breast cancer cell lines or untransfected CHO cells.

TABLE 1

CELL LINE	[PTHrP] (pM/10^5 cells/24 hr)	ESTROGEN RECEPTOR
MDA-MB-231	5.6±0.4	Negative
ZR-75-30	<0.3	Negative
T-47D	<0.3	Positive
MCF-7	<0.3	Positive
RWGT2	210±11	Unknown
CHO-K1	<0.3	Unknown
CHO/PTHrP-(1-141)	56±3.1	Unknown
CHO/PTHrP-(1-141)-AS	<0.3	Unknown

This work represents part of specific aim 1a. Currently, other 10 other breast cancer cell lines are being screened for PTHrP production. Once screening is complete, estrogen regulation of PTHrP mRNA will be performed as proposed in specific aim 1b.

Experiment 1

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 metastasis model. As MDA-MB-231 was 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 as described under experiment 1. PTHrP production *in vitro* by MDA-MB-231 and RWGT2 is illustrated in figure 3.

Results of Ca^{++} measurement are illustrated in figure 4. On the left portion of figure 4, mice inoculated with local intramuscular tumors had similarly normal calcium concentrations at baseline. Four weeks later, at sacrifice, the mice bearing the high-PTHrP-producing RWGT2 tumors developed marked hypercalcemia while the mice bearing MDA-MB-231 remained normocalcemic. On the right portion of figure 4, mice inoculated with tumor cells via the left cardiac ventricle had normal calcium concentrations at baseline. At the time of sacrifice, mice with osteolytic lesions due to RWGT2 were minimally, but significantly hypercalcemic when compared with the mice bearing osteolytic lesions due to MDA-MB-231. Note that the hypercalcemia in the metastatic RWGT2 group was mild compared with mice bearing localized RWGT2 tumors.

Plasma PTHrP concentrations, are illustrated in figure 5. On the left portion of figure 5, at baseline mice inoculated with local, nonmetastatic RWGT2 or MDA-MB-231 tumors had similarly low plasma PTHrP concentrations at baseline. Four weeks later, at sacrifice, hypercalcemic mice bearing local RWGT2 tumors had a significant increase in plasma PTHrP concentrations while normocalcemic mice bearing local MDA-MB-231 tumors did not differ from baseline. On the right portion of figure 5, baseline plasma PTHrP concentrations were similarly low in both RWGT2 and MDA-MB-231 groups inoculated via the left cardiac ventricle. At sacrifice, only the mice bearing osteolytic lesions due to RWGT2, which were mildly hypercalcemic, had a significant increase in plasma PTHrP concentrations. This increase was significantly less than those animals bearing local intramuscular RWGT2 tumors.

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 the above described immunohistochemical technique for PTHrP. Both MDA-MB-231 and RWGT2 in muscle and in bone stained positively for PTHrP.

Experiment 2

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. Figure 6 illustrates PTHrP concentration as measured in conditioned media from the parent MDA-MB-231 cell line and stably transfected MDA-MB-231 clones expressing PTHrP in either the sense (MDA/PTHrP-1) or antisense (MDA/PTHrP-AS) orientation. 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. Details of methodology are described above. Calcium and PTHrP concentrations in tumor-bearing mice are illustrated in figure 7. Calcium concentrations, illustrated in the left portion of figure 7, were similar in all groups at baseline. At the time of sacrifice, calcium concentrations were minimally, but significantly, increased over baseline only in mice inoculated with the high-expressing MDA/PTHrP-1 clone. Plasma PTHrP concentrations, illustrated in the right portion of figure 7, were similarly low at baseline and at sacrifice in all three groups. The dashed line indicates the lower limit of detection in this assay (0.3 pM).

The graph in figure 8, on the left portion, illustrates the number of osteolytic lesions, in each group, evident on radiographs at 3 weeks. 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. Representative radiologic and histologic appearance of osteolytic lesions are shown in the insets on the right portion of figure 8. Histologic sections of all long bones with osteolytic lesions from all groups revealed tumor adjacent to osteoclasts resorbing bone. Survival in mice with metastases is illustrated in figure 9. 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. Figure 10 illustrates the growth curve for MDA/PTHrP-1, MDA/PTHrP-AS and MDA-MB-231. No differences in cell growth were observed during the 8 day period in which the cells were in culture.

CONCLUSIONS

These results demonstrate that 1 of 4 breast cancer cell lines screened (MDA-MB-231) secretes PTHrP *in vitro* and this was not related to the presence of the estrogen receptor. Clearly, more breast cancer cell lines (both estrogen receptor positive and negative) need to be screened before any relationship between PTHrP expression and estrogen receptor status can be significantly determined. Presently, further screening is underway and estrogen regulation of PTHrP expression in breast cancer cells will be studied during the next year.

In vivo data from experiment 1 demonstrate that tumor-mediated osteolysis is associated with osteoclastic bone resorption at sites of tumor involvement. Hypercalcemia is mild compared with humoral hypercalcemia and occurs when tumors express large amounts of PTHrP and when plasm PTHrP concentrations are increased, as demonstrated with the RWGT2 tumor. Thus, PTHrP can cause hypercalcemia when produced at sites distant from bone and locally by tumor in bone. Localized osteolysis mediated by PTHrP may occur without hypercalcemia as immunohistochemical staining of MDA-MB-231 in bone demonstrated local PTHrP production. However, these results are not conclusive and further experiments of this nature will be performed with anti-PTHrP neutralizing antibodies. These data do suggest that different tumor syndromes may be associated with PTHrP production and depend on the tumor location and degree of PTHrP expression.

In vivo data from experiment 2 demonstrate that PTHrP overexpression in a breast cancer cell line is associated with an increased number of osteolytic metastases, hypercalcemia without an increase in plasma PTHrP concentration, and decreased survival. The effect of PTHrP to cause hypercalcemia in this model appears to be local as plasma PTHrP concentrations were not increased over the nontumor bearing state.

These data support the clinical findings that PTHrP, as a bone resorbing factor, may contribute to the ability of breast cancer to grow in bone. PTHrP-expressing breast cancer cells lodged in bone locally stimulate osteoclastic bone resorption and growth factors present in bone matrix, such as TGF-β, are released into the bone microenvironment. As referenced in the background, TGF-β enhances PTHrP expression in some cancer cells and other growth factors may stimulate their proliferation as well. This can initiate a cycle in which tumor cells express more PTHrP, further increase osteoclastic bone resorption and become established tumors in bone. The findings in which PTHrP expression by breast cancer cells enhance the development of osteolytic metastases have important implications in the prognosis of women with newly diagnosed breast cancer as well as in the treatment of breast cancer metastatic to bone. Therapeutic modalities designed to break this cycle in bone may improve the morbidity associated with advanced metastatic breast cancer to bone and inhibitors of osteoclastic bone resorption may decrease the development of bone metastasis in women with PTHrP positive primary breast tumors.

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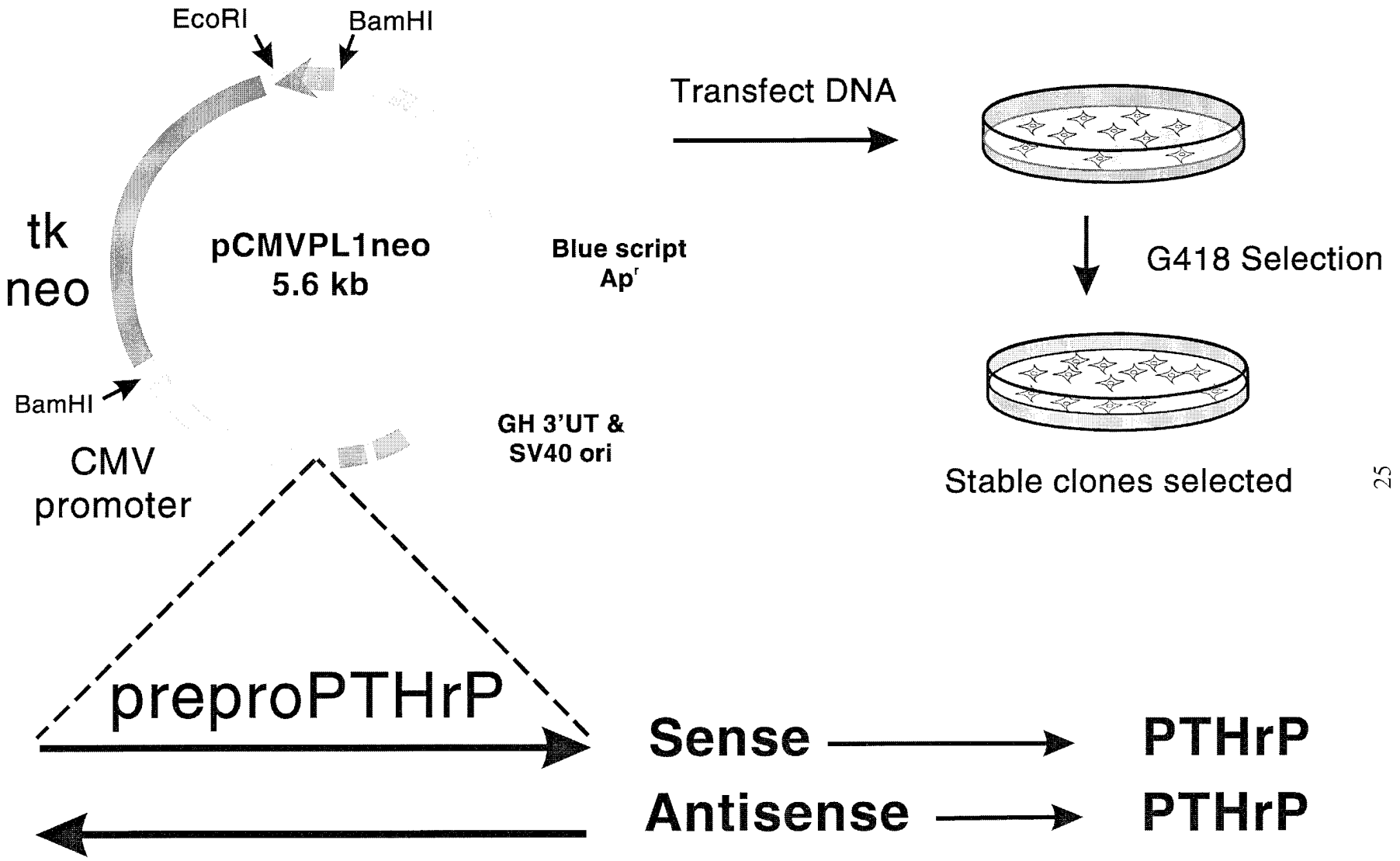
APPENDIX

Figures 1 - 10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BamHI
tk
neo
pCMVPL1neo
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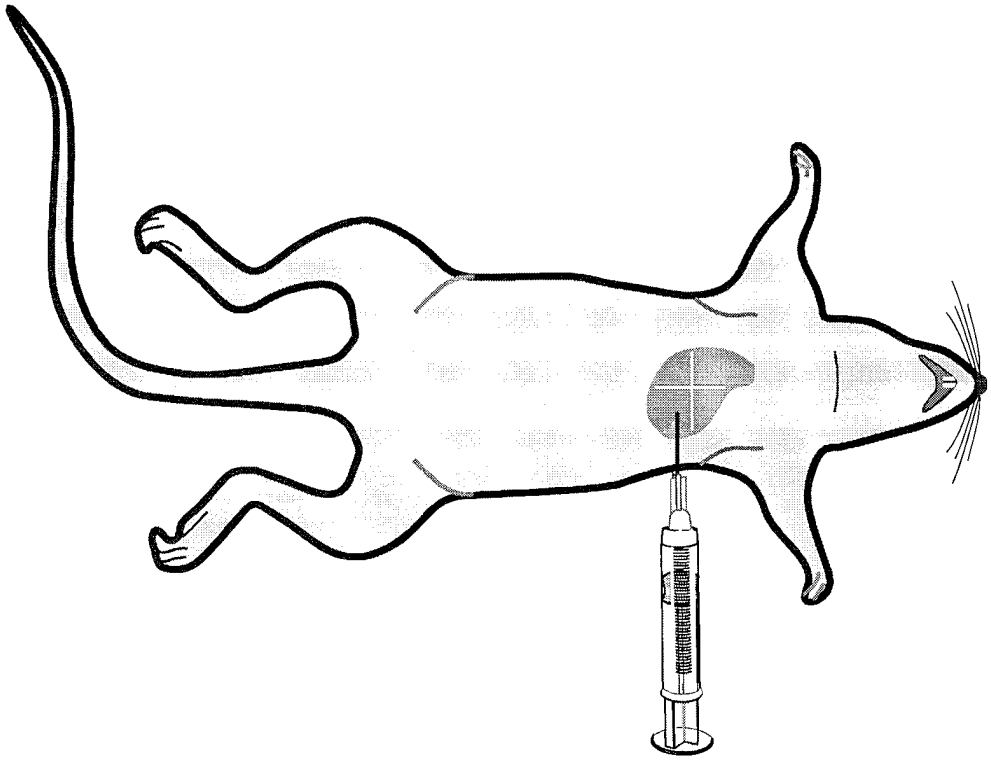
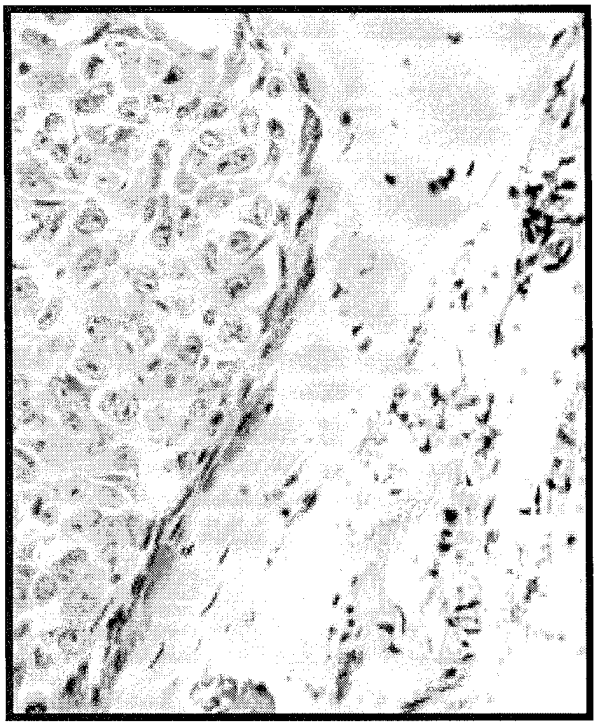
Blue script
Ap^r
BamHI
CMV
promoter
GH 3'UT &
SV40 ori
preproPTHrP
Transfect DNA
G418 Selection
Stable clones selected
Sense
PTHrP
Antisense
PTHrP

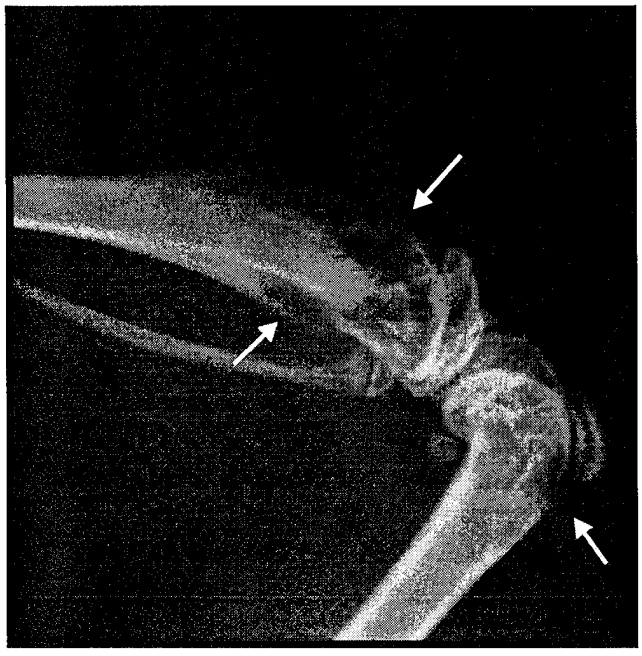
FIGURE 1

FIGURE 2



Bone Metastasis Model





[PTHrP] in 24-hour Conditioned Media

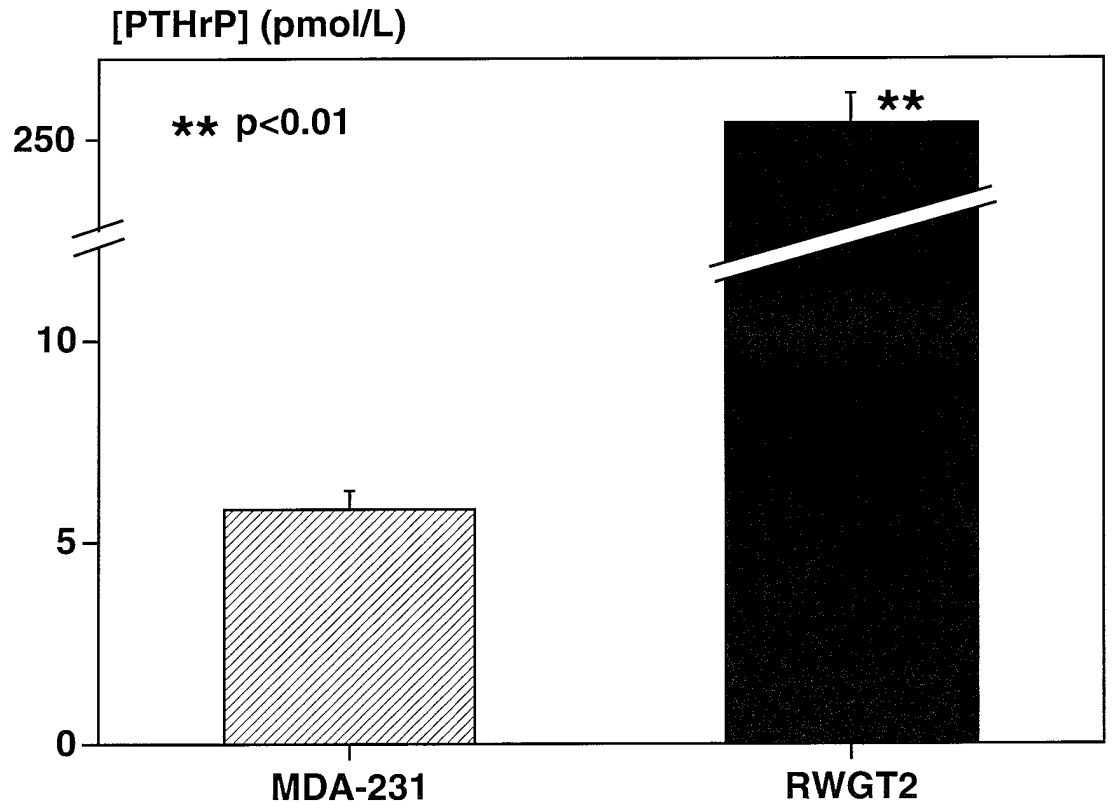


FIGURE 3

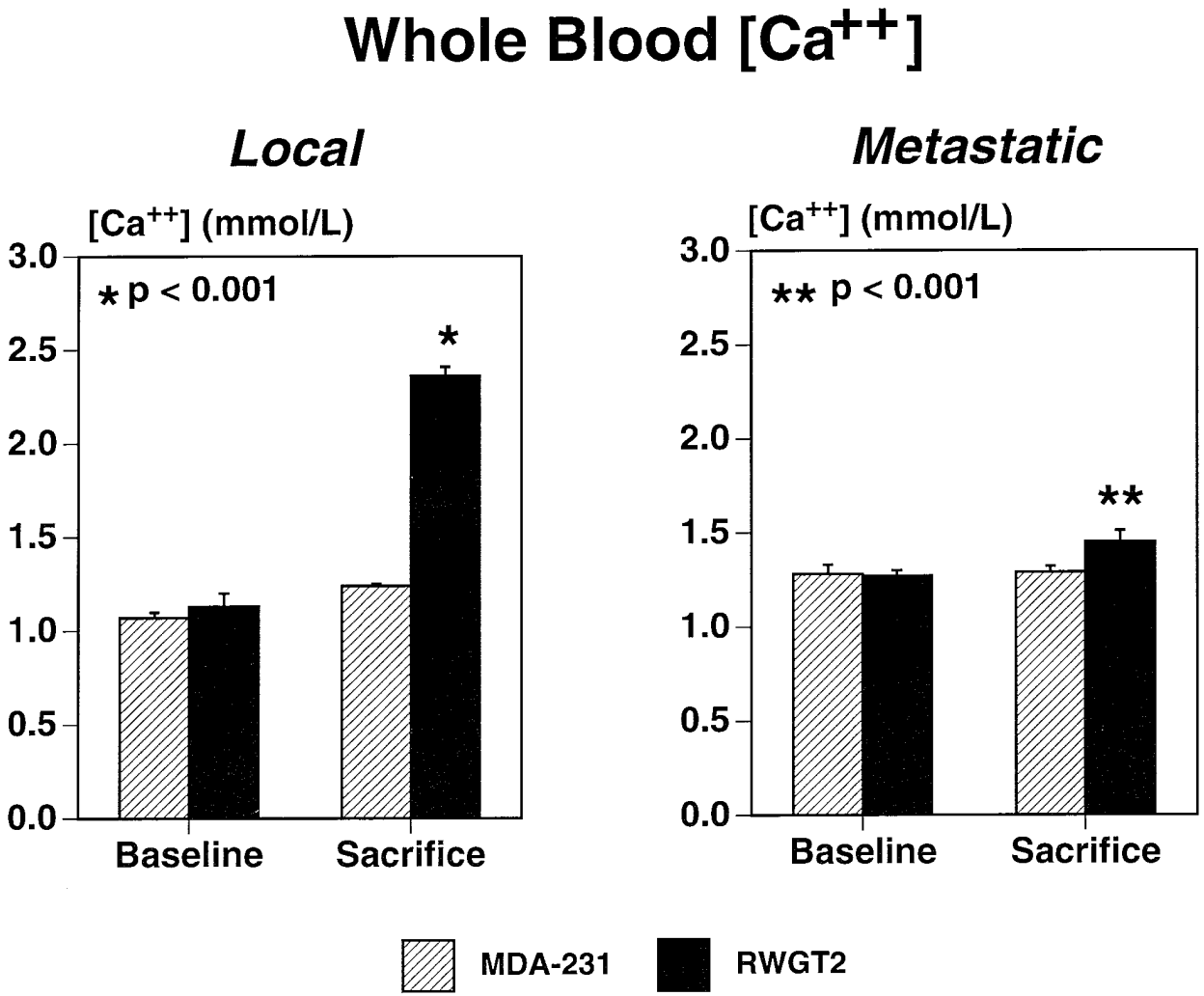

Whole Blood [Ca++]
Local
Metastatic
[Ca++] (mmol/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
* p < 0.001
** p < 0.001
*
**
Baseline
Sacrifice
MDA-231
RWGT2

FIGURE 4

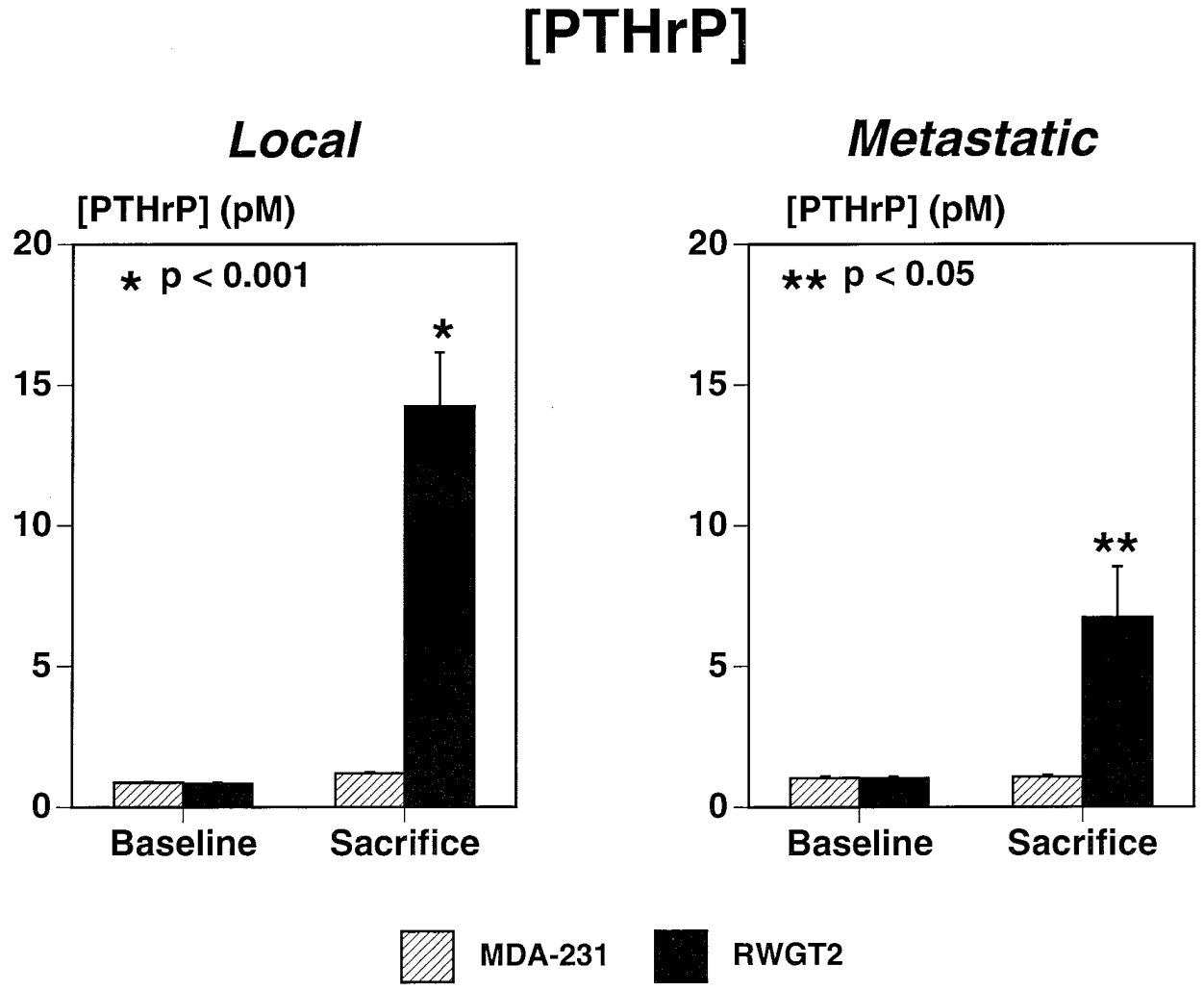

[PTHrP]
Local
[PTHrP] (pM)
20
15
10
5
0
* p < 0.001
*
Baseline
Sacrifice
Metastatic
[PTHrP] (pM)
20
15
10
5
0
** p < 0.05
**
Baseline
Sacrifice
MDA-231
RWGT2

FIGURE 5

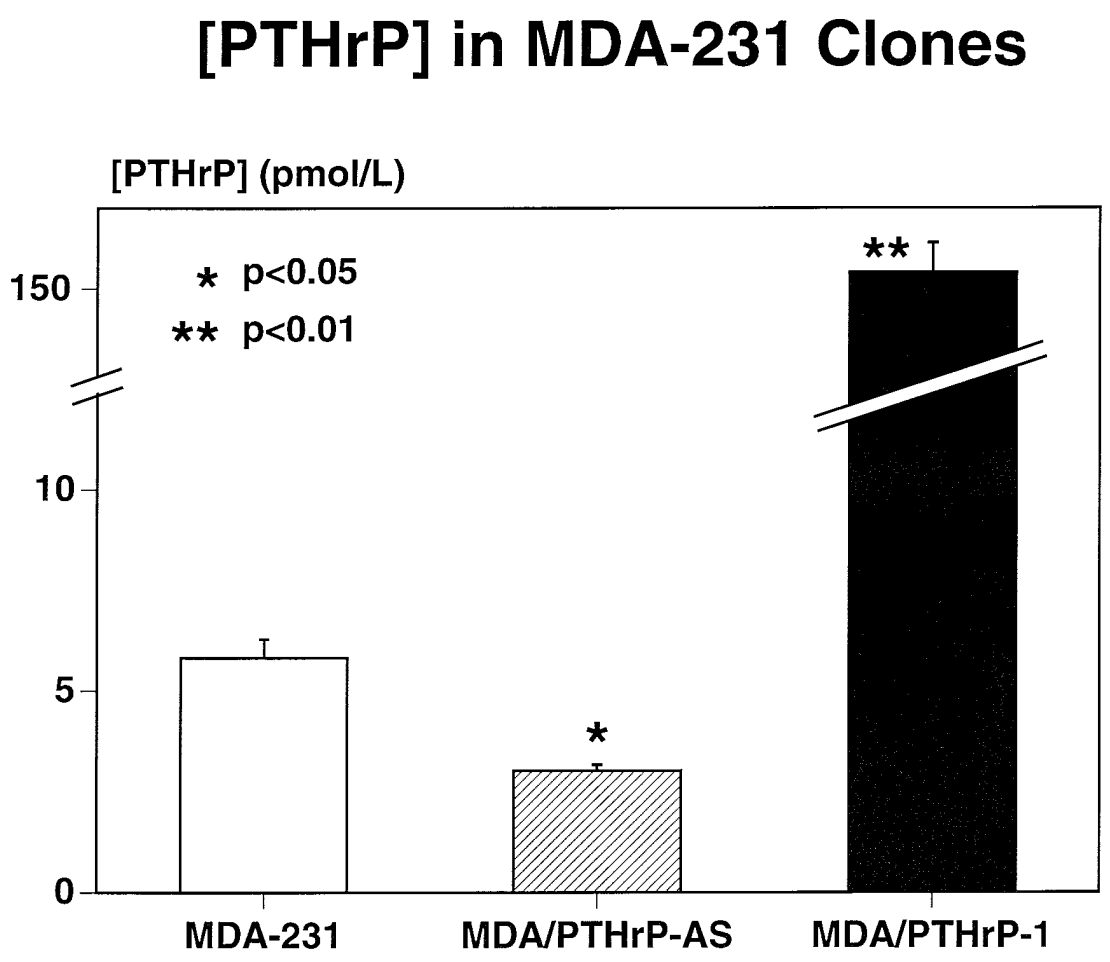

[PTHrP] in MDA-231 Clones
[PTHrP] (pmol/L)
* p<0.05
** p<0.01
150
10
5
0
**
*
MDA-231
MDA/PTHrP-AS
MDA/PTHrP-1

FIGURE 6

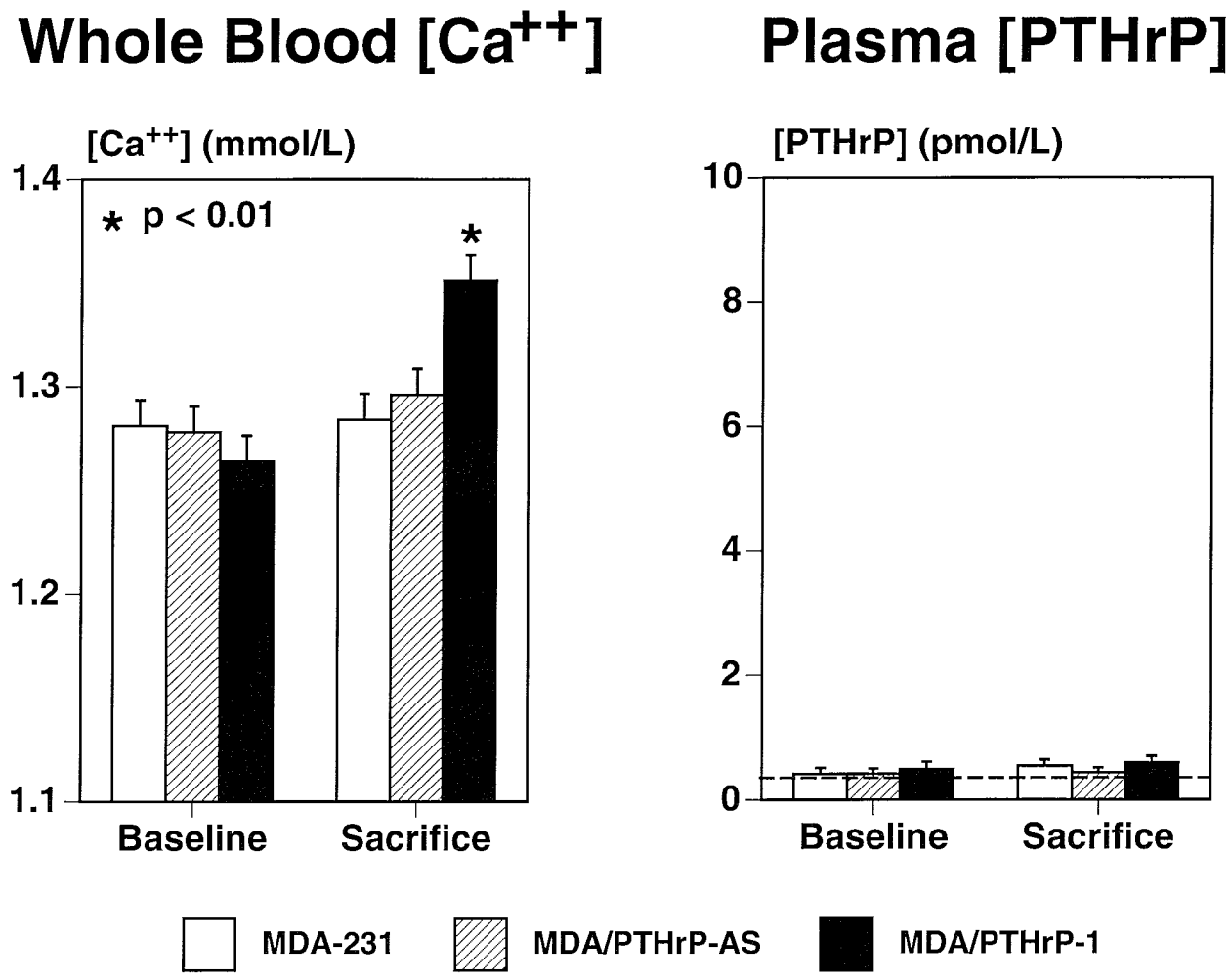
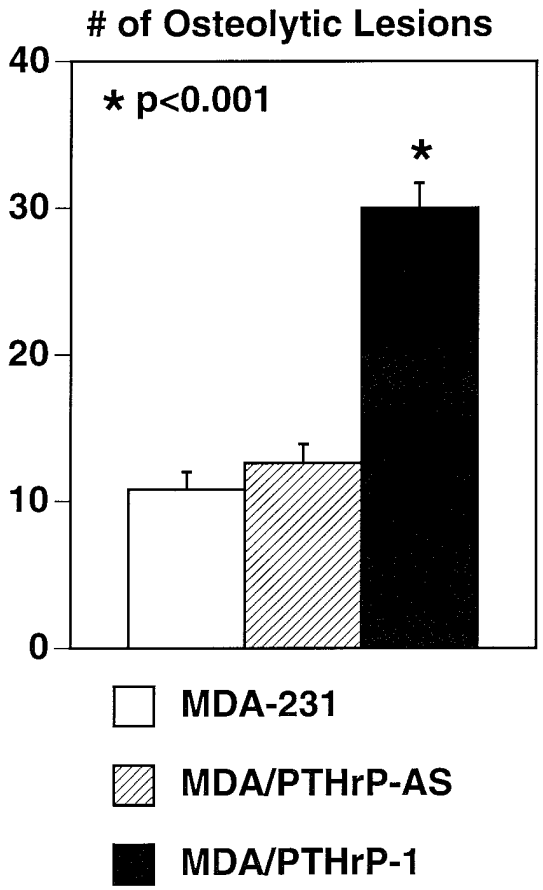

Whole Blood [Ca++]
Plasma [PTHrP]
[Ca++] (mmol/L)
1.4
1.3
1.2
1.1
* p < 0.01
*
Baseline
Sacrifice
[PTHrP] (pmol/L)
10
8
6
4
2
0
Baseline
Sacrifice
MDA-231
MDA/PTHrP-AS
MDA/PTHrP-1

FIGURE 7

Osteolytic Lesions at 3 Weeks



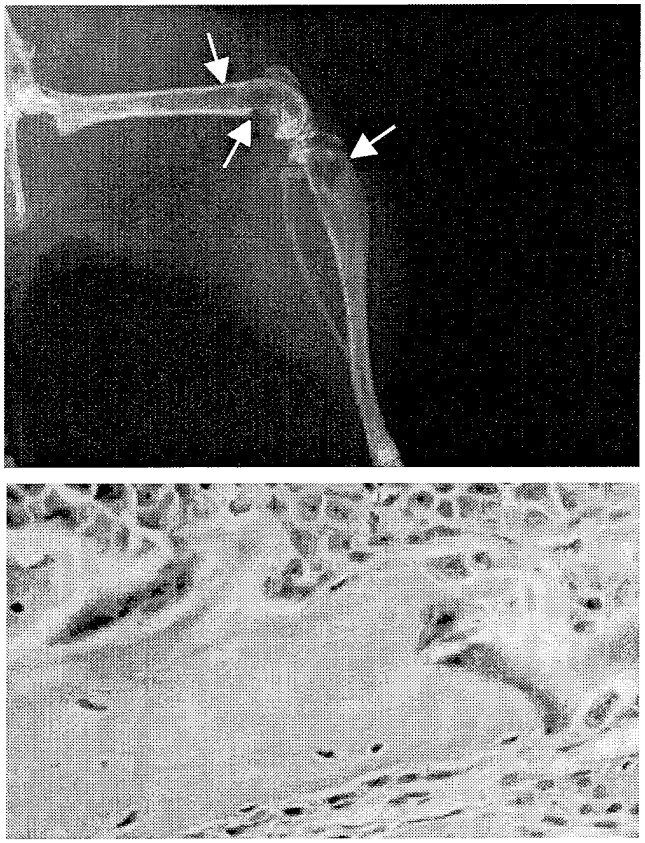


FIGURE 8

Survival in Mice with Osteolytic Lesions

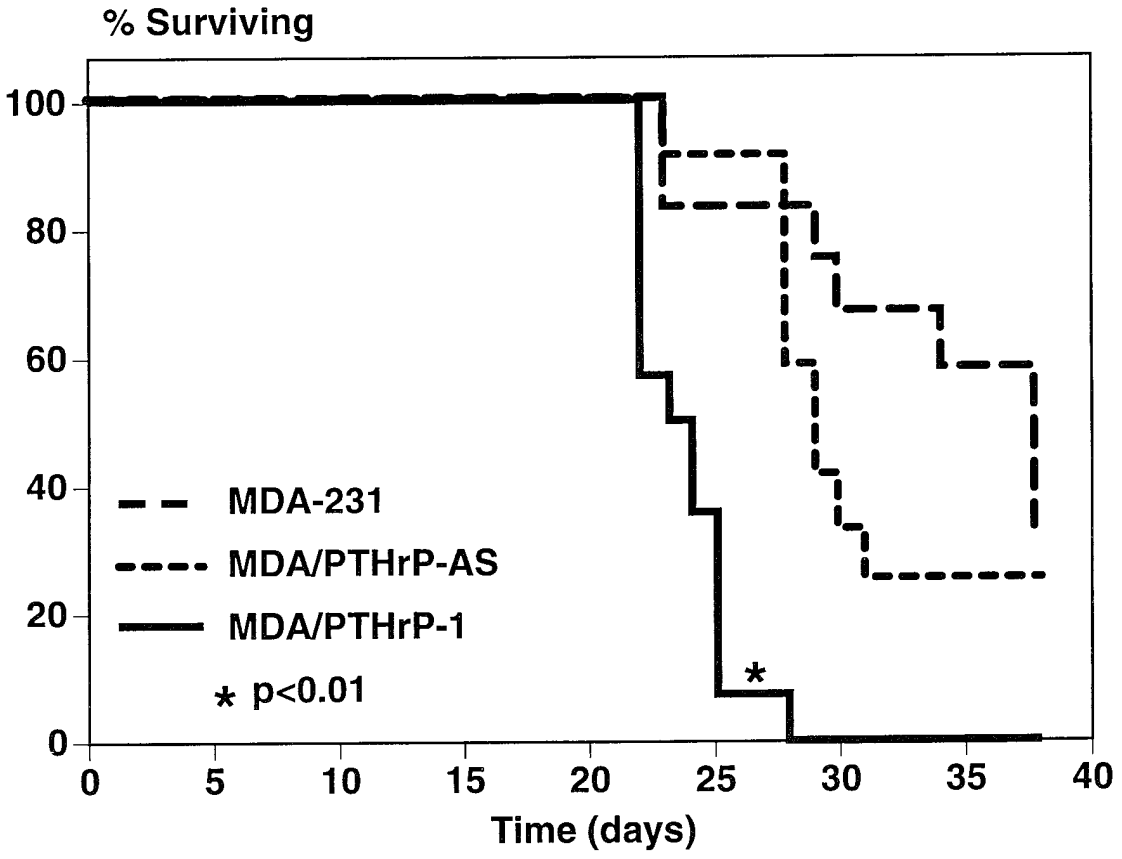


FIGURE 9

Cell Growth of PTHrP-Expressing MDA-231 Breast Cancer Clones

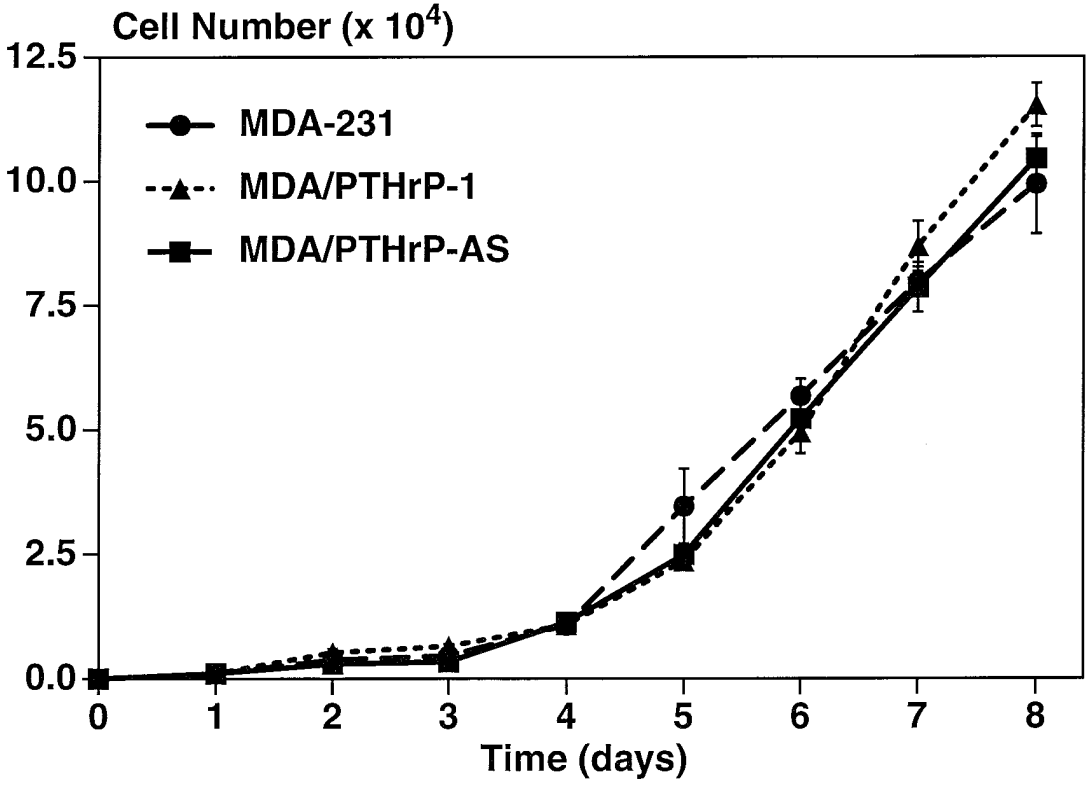


FIGURE 10