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TITLE: The Distribution, Levels, and Relevance of the Interleukin-1 Family of Cytokines and Receptors in Human Breast Carcinoma-Induced Osteolysis

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13. ABSTRACT (Maximum 200 Words)

Bone metastasis in human breast carcinoma (HBC) occurs in 83% of patients with advanced disease. HBC bone metastasis causes degeneration of the bone matrix (osteolysis), hypercalcemia, pathologic fracture, and nerve-compression syndrome. The pathophysiology of human breast carcinoma-induced osteolysis (HBC-IO) involves an increase in the number and activity of osteoclasts within the HBC metastatic lesion. We examined the expression of the IL-1 family of cytokines and receptors and IL-8 in HBC-IO using archival human samples (mean age, 52yrs; age range, 34-83yrs; no prior radiation to site) and immunohistochemistry. We observed IL-1 and IL-8 expression by HBC cells and IL-1Receptor I expression on osteoclasts. These data suggest that HBC-derived IL-1 is an important mediator of human breast cancer-induced osteolysis and supports our hypothesis: *1.* IL-1 can activate osteoclastogenesis, promote osteoclast (OC) activation and osteolysis *via* paracrine induction of IL-1Receptors on osteoclasts. *2.* IL-1 can promote tumor progression by autocrine induction and subsequent activation of IL-8. *3.* IL-8 expressed by HBC cells can support tumor growth and progression by stimulating angiogenesis through IL-8 Receptors expressed on vascular endothelial cells. This study suggests that IL-1 may be an important mediator of HBC pathophysiology and therefore, a potential target for therapeutic intervention.

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Human breast cancer, cytokine networks, bone metastasis

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A. INTRODUCTION:

Human breast carcinoma-induced osteolysis (HBC-IO). Bone metastasis in human breast carcinoma (HBC) occurs in 83% of patients with advanced disease. Unlike most other types of cancer, breast cancer has a predilection for spreading to the bone, and bone metastasis is a major cause of increased pain, morbidity and mortality. Clinically, bone metastasis causes degeneration of the bone matrix (osteolysis), hypercalcemia, pathologic fracture, and nerve-compression syndrome. The pathophysiology of HBC-IO is not well understood. It is generally thought that HBC-IO is related to an increase in the number and activity of bone resorbing cells, the osteoclasts, at the site of the HBC metastatic lesion. These observations strongly support the involved of cell-to-cell interactions and cytokine networks in HBC-IO. IL-1 has been demonstrated in bone resorption and IL-1 has been shown to be expressed by HBC cells, however a major gap exists in our understanding of processes that occur in HBC-IO and the role of tumor-cell derived IL-1. ***Rationale/purpose:*** Since IL-1 expression has been correlated with HBC aggressiveness and IL-1 is a known activator of osteoclasts, we propose to study the levels and distribution of the IL-1 family of cytokines and receptors in HBC-IO using patient tissue samples. ***Objectives:*** 1. Demonstrate the presence, distribution and levels of the IL-1 family of cytokines and receptors [IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra), IL-1 RI, and IL-1RII] within the HBC-IO microenvironment using immunohistochemistry and ELISA, 2. Demonstrate the presence, distribution and levels of osteogenic activators/markers of osteolysis (RANKL, PTHrP and OPG) within the HBC-IO microenvironment using immunohistochemistry and ELISA, 3. Quantitate disease/lesion severity using histomorphometry (bone density, osteoclast number and distribution, tumor size and distribution), and 4. Correlate 1, 2, and 3 as described above with clinical diagnosis (primary benign bone lesions *vs.* primary malignant *vs.* bone metastasis from breast carcinoma).

B. BODY:

TASK 1. **Collect patient surgical samples.**

This study uses tissue from patients that have undergone surgical procedures related to orthopaedic oncology disease. The samples were collected under IRB except protocols and consist of archival samples or surgical discard samples.

1a. Research Accomplishments. The following type and numbers of samples have been obtained:

- Normal marrow.
  a. No bone marrow samples have been collected since these are not available as archival or surgical discard samples and in retrospect are not important to the quality or goals of this project.
- Primary benign bone tumors (giant cell tumor, non-ossifying fibroma and enchondroma).
  a. Giant cell tumors: 13 archival paraffin embedded 20 sections/ sample, 3 snap frozen.
  b. Enchondroma: 11 archival paraffin embedded 20 sections/ sample, 0 snap frozen.
- Primary malignant bone tumors (Ewing's sarcoma, malignant fibrous histiocytoma and osteogenic sarcoma).
  a. Osteosarcoma: 10 archival paraffin embedded 20 sections/ sample, 8 snap frozen
  b. Malignat fibrous histiocytoma: 6 archival paraffin embedded 20 sections/ sample, 0 snap frozen.
- Bone metastasis (breast origin). 23 archival paraffin embedded 20 sections/ sample, 2 snap frozen.

In support of obtaining human samples we have completed IRB applications and established clinical collaborations with the following institutes and individuals:

- University of Connecticut Health Center, Farmington, CT, Melinda Sanders, M.D.
- Hartford Hospital, Hartford, CT, Dr. Andrew Ricci, M.D.
- Yale University School of Medicine, New Haven CT, Dieter Lindskog, M.D.
- University of New Mexico, Robert Quinn, M.D.
- Cooperative Human Tissue Network, CHTN

1b. Problems and Solutions. This project examines the expression of cytokines in human patient samples using immunoassays such as immunohistochemistry (IHC) and ELISA. As such, we require microscope tissue sections for IHC and snap frozen pieces of tissue for ELISA. While we have been successful in obtaining sufficient samples for IHC we do not have enough snap frozen surgical discards to quantitate cytokine levels by ELISA. My collaborator, Dr. Robert Quinn, M.D., the only orthopaedic oncologist in the greater Hartford area, resigned and has established himself in New Mexico in 2004. We have re-established our research collaboration infrastructure, have an IRB (exempt) application in the final stages of approval, and expect to be obtaining samples in support of this work in the near future. I am also working with Dr. Dieter Lindskog, Yale University School of Medicine and have written an IRB (exempt) to obtain tissue. I have also established a protocol with Cooperative Human Tissue Network (CHTN), but have not found then to be a valuable resource for the specific samples I require. Despite my enthusiasm for this project, it still takes time to get the paperwork approved and get collaborators to contribute. The samples for this part of the project represent one part of the grant and other contingencies (the IL-8 studies) are in place to keep the research flowing. We have requested a no cost extension and will continue our search for snap frozen tissues.

Antigen	Isotype	Company	Product #
IL-1α	rabbit	Endogen	P420A
IL-1α	mouse	Endogen	M420A
IL-1α	mouse	Endogen	M421A
IL-1α	mouse	Biosource	AHC0912
IL-1β	rabbit	Endogen	P420B
IL-1β	mouse	Endogen	M421B
IL-1β	Goat	R + D	AF-201-
IL-1β	mouse	abcam	ab8320
IL-1ra	rabbit	abcam	ab2573
IL-1ra	rabbit	Endogen	P3001
IL-1R1	rabbit	SC	SC 687
IL-RII	mouse	R&D	MAB263
IL-8	rabbit	Endogen	P801
IL-8	Goat	R + D	AF-208-
IL-8	mouse	Endogen	M801
IL-8RA	rabbit	SC	SC-988
IL-8RB	mouse	SC	SC-7304
IL-8RB	mouse	R + D	MAB331
PTHrP	mouse	IDS	AE-0502
osteoclast	mouse	IDS	AE-4002
calcitonin receptor	mouse	Serotec	MCA2191
cathepsin K	chicken	IDS	AE-3002
(-)control	rabbit Ig	Pierce	31887
(-)control	mouse Ig	Pierce	31878

Table 1. Panel of 20/22 anti-human antibodies we have evaluated to examine HBC bone metastasis in de-calcified bone samples.

TASK 2. **Determine the distribution of the IL-1 family of cytokines and receptors in primary benign, primary malignant and breast ossous-metastatic samples.**

The major goal of this program was to determine if the pro-inflammatory cytokine, Interleukin-1 (IL-1), family of agonists, antagonists and receptors are present in the breast cancer osseous metastatic microenvironment. Furthermore, expression is to be compared to primary bone benign and malignant tumors using IHC.

2b. Research Accomplishments. Since we are exploring a new area where no published research has been done we first needed to define as establish IHC protocols IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra), IL-1Receptor I (IL-1RI) and IL-1RII using paraffin embedded samples that are de-calcified. To accomplish this we evaluated different methods of antigen retrieval using 1. citrate buffer with various heat treatments, 2. 4NHCl, and 3. DeCal Retrieval (BioGenix). Additionally we examined 20/22 different antibodies (Table 1) with these methods. The second goal of this task was to determine the expression of IL-1 by cell type and relative staining intensity. The results are summarized in table 2 and figure 1.

	IL-1β			IL-1Receptor I			IL-8		
	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)
Tumor Cells	13/16 (81%)	82%	2.3	13/16 (81%)	62%	2.0	14/17 (82%)	34%	1.3
Osteoclasts	0/16 (0%)	0%	0	15/16 (94%)	74%	2.8	0/17 (0%)	0%	0

Table 2. Immunohistochemical expression of the IL-1 family and IL-8 in HBC osteolytic lesions. No expression of IL-1RII, or IL-1Ra was observed.

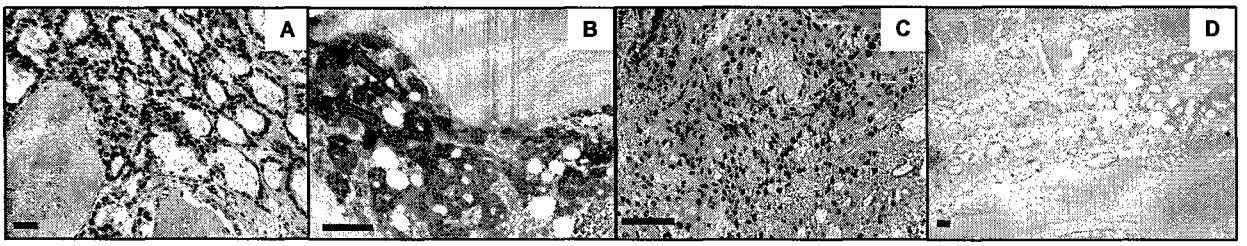

Figure 1. Immunhistochemical assay for the expression of: (a) IL-1b, (b) IL-1 RI [arrow points to staining osteoclasts], (c) IL-8 and (d) Ig negative control in human breast cancer bone metastasis. Bar = 100mm

2a Problems and Solutions. We wasted one month using the anti-osteoclast Ag antibody supplied from IDS. The technical support documents clearly support the use of this product for both formalin fixed paraffin embedded (FFPE) tissue and frozen section (OCT). The U.S. supplier assured us that it worked with FFPE and would contact technical support in the U.K. regarding this issue. The U.K. division was on "holiday" for that month and because of the importance to obtain this data for an international meeting we proceeded to trouble shoot the assay. The results from our work and later substantiate by the manufacturer, was that IDS anti-osteoclast antigen AE-4002 does not work with FFPE and the company has removed this application to it's documentation. To resolve the issue of identifying osteoclasts we have obtained anti-Calcitonin receptor (Serotec MCA2191) and Cathepsin K (IDS AE-3002). We have yet to evaluate these antibodies and would like to use them in a double-stain assay (BioCare) with IL-1 RI.

TASK 3. **Determine the distribution of cytokine and cytokine receptors that are thought to be osteogenic activators/markers of osteolysis in primary benign, primary malignant and breast ossous-metastatic samples.** This part of the study is to correlate the expression of IL-1 with receptor activator of NF-kappa B ligand (RANKL), RANKL inhibitor, osteoprotegerin (OPG) and parathyroid hormone related protein (PTHrP).
3a. Research Accomplishments. We found that the HBC tumor express PTHrP with less intensity than IL-1.
3b. Problems and Solutions. We have not evaluated the expression of RANKL or OPG. Recently RANKL was evaluated be another research group. No evidence of RANK-L expression was observed on breast cancer cells, (*personal communication*: Dr. Bhatia, University of Connecticut Health Center).

TASK 4. **Determine the levels of cytokines and receptors of the IL-1 family and osteogenic activator/markers of osteolysis.** This part of the study is to quantitate the levels of the IL-1 family of cytokines and receptors from tissue homogenates of snap frozen tissues.
4a. Research Accomplishments. Although we have obtained a small number of samples, we have not evaluated any tissue homogenates by ELISA.
4b. Problems and Solutions. We have, to date, not been able to obtain sufficient numbers of samples to complete this task. Details are described in section 1c above.

TASK 5. **Determine the relevance of cytokine and cytokine receptor expression data from immunohistochemistry and ELISA.** This part of the study is ongoing and requires completion of IL-1 expression in benign and malignant bone tumors as well as ELISA evaluation.

C. KEY RESEARCH ACCOMPLISHMENTS:

- **Established the methods and protocols for IHC evaluation of cytokines and receptors in pathological bone de-calcified FFPE tissue sections.**
- **Demonstrated the expression of IL-1b by human breast cancer cells in osseous metastatic lesions.**
- **Demonstrated the expression of IL-1R1 by human breast cells and osteoclasts in osseous metastatic lesions.**
- **Demonstrated lack of expression of the IL-1 antagonists (IL-1ra) and IL-1 RII in osseous metastatic lesions.**
- **Demonstrated expression of the pro-tumorogenic (growth and angiogenesis factor) factor, IL-8, by human breast cancer cells in osseous metastatic lesions.**

D. REPORTABLE OUTCOMES:

D1. Professional:

In April of 2004, I was promoted to Assistant Professor of Orthopaedic Surgery.

D2. Abstracts:

1. **A.G. Pantschenko,** R. Naujoks, R. H. Quinn, M. Sanders, G. Gronowicz. Cellular Cooperativity *via* Proinflammatory Cytokine Networks in the Human Breast Carcinoma Bone Metastatic Microenvironment. Skeletal Complications of Malignancy IV. April 28-30, 2005 Natcher Conference Center, NIH, Bethesda, MD (Attachment 1).
2. **A.G. Pantschenko**, R. Naujoks, R. H. Quinn, M. Sanders, G. Gronowicz. Interleukin-1 Expression in Human Breast Cancer Bone Metastasis: a Newly Recognized Pathway in Breast Cancer-induced Osteolysis. 2004. American Society for Bone and Mineral Research 26th Annual Meeting. JBMR vol 19 suppl 1 ppS227 SU094. (Attachment 2).

D3. Funding Applied for Based on Work From this Award:

1. **Title: Human Breast Cancer Bone Metastasis: Unraveling the Tumor Microenvironment using Laser Capture Microdissection and Gene Array Analysis.**
   PI: Alexander G. Pantschenko, Ph.D. **Agency:** Sidney Kimmel Foundation
   Type: Research **Period:** 7/1/05 – 6/30/07
   Compare and contrast gene expression in the osteolytic vs. non-osteolytic breast cancer osseous metastatic microenvironment.
2. **Title: Breast Cancer-derived Interleukin-1 in Osseous Metastasis.**
   PI: Alexander G. Pantschenko, Ph.D. **Agency:** NCI
   Type: KO1 Career Development **Period:** 7/1/05 – 6/30/10
   Determine the role of IL-1 in breast cancer osteolytic disease using in vitro models and a xenograft animal model with IL1 agonist, antagonist and receptor transfected human breast cancer.
3. **Title: Breast cancer osseous metastatic disease: understanding the role of proinflammatory cytokines and receptors in tumor cell modulation of the bone microenvironment.**
   PI: Alexander G. Pantschenko, Ph.D. **Agency:** Komen Fundation
   Type: Research **Period:** 4/1/05 – 3/31/06
   Understand the contribution of proinflammatory cytokines (IL-1, IL-8, & TNF) in osteoclast activation and tumor angiogenesis using xenograft model of breast cancer osseous metastasis.
4. **Title: Validation of the *In Vivo* Mouse Xenograft Models to Determine the Role of Interleukin-1 (IL-1) in Breast Cancer-induced Osteolysis.**
   PI. Alexander G. Pantschenko **Agency:** UCHC Womens' Center
   Type: Pilot **Period:** 7/1/05- 6/30/05
   Evaluate the currently used xenograft mouse model of breast cancer osseous metastasis for expression of pro-inflammatory cytokines.

5. **Title: Establishing the Xenograft Model: Pro-Inflammatory Cytokines as Mediators of Tumor Progression and Osteolysis in Breast Cancer Bone Metastasis.**
   PI: Alexander G. Pantschenko, Ph.D. **Agency:** Dept. of Defense Breast Cancer Research Program
   Type: Concept Award **Period**: 9/30/05-9/29/06
   The major goal of this project is to characterize the expression and role of Interleukin-1 (IL-1) and IL-1 receptors in breast cancer bone metastasis with the murine xenograft model.
6. **Title: *In Vitro* Evaluation of Therapeutic Touch on Human Breast Cancer Growth, Invasiveness and Expression of Tumor Progression Factors.**
   PI: Alexander G. Pantschenko, Ph.D. **Agency:** Dept. of Defense Breast Cancer Research Program
   Type: Concept Award **Period**: 9/30/05-9/29/06
   This project is an in vitro evaluation of the affect of therapeutic touch on human breast cancer aggressiveness.
7. **Title: *In Vivo* Bioluminescent Imaging to Study Breast Cancer Osseous Metastatic Disease.**
   PI. Alexander G. Pantschenko **Agency:** UCHC Center for Musculoskeletal Research
   Type: Pilot **Period:** 7/1/05- 6/30/05
   Establish the techniques and infrastructure for bioluminescent imaging and luciferase based assays in animal models of tumor growth and metastasis.

E. CONCLUSIONS:

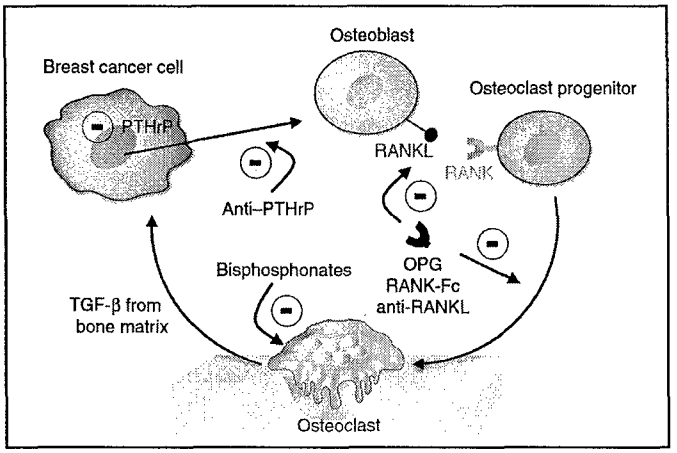

Figure 2. Proposed Role of PTHrP in Breast Cancer Osteolysis. *Adapted from T. John Martin J. Clin. Invest. 110:1399-1401 2002.*

In efforts to elucidate the mechanism of HBC-induced osteolysis a great deal of attention has been give to parathyroid hormone-related protein (PTHrP). PTHrP has been detected by immunohistochemistry in 80-90% of patients with breast cancer bone metastasis [1, 2] and increases the number of osteolytic lesions the MDA-MB-231 xenograft animal model [3]. Further animal experiments demonstrate that MDA-MB-231 cells alone or cells isolated from breast cancer samples do not express RANK-L mRNA. However, when HBC cells were co-cultured with stromal cells or osteoblasts, receptor activator of NF-kappa B-Ligand (RANK-L) mRNA was expressed and the RANK-L decoy receptor, osteoprotegerin (OPG) mRNA expression was decreased. These experiments suggest that interactions between breast cancer and stromal or osteoblastic cells induce osteoclastogenesis *in vitro* through modulating RANKL expression [4]. Therefore, PTHrP does not directly influence osteoclasts, but supports osteoclastogenesis indirectly through the upregulation of RANKL on osteoblasts. Additionally, during bone resorption, transforming growth factor beta (TGFβ) is released from the bone matrix and stimulates breast carcinoma cells to produce additional PTHrP, which in turn, perpetuates osteoclast-mediated HBC-IO (Figure 2). Although this model represents the current paradigm, it primarily derived from animal and tissue culture studies. Recent human studies dispute this mechanism for the activation of osteoclasts. Examining various osteolytic human bone tumors for RANK-L expression, Good *et. al.* were unable to show expression in breast metastasis [5]. In a larger study of 42 samples of human breast cancer bone metastasis, no evidence of RANK-L expression was observed on breast cancer cells, however osteoblasts were not evaluated (*personal communication*: Dr. Pardieb Bhatia, University of Connecticut Health Center). Clearly, other models of HBC-induced osteolysis must be explored.

We are the first to demonstrate the expression of Interleukin-1 (IL-1) by human breast carcinoma (HBC) cells and the expression of the IL-1Receptor-I on osteoclasts at the site of osteolysis in human bone samples (*appendix)* [6]. Furthermore, we have shown with cell lines that IL-1, acting through it's receptor on HBC cells,

regulates the autocrine activation of the tumor cell mitogen and angiogenesis factor, IL-8 [7, 8]. Based on our work and the literature, we hypothesize that HBC cells can directly regulate osteoclasts (and therefore osteolysis) by the IL-1 network and that IL-1 up-regulates HBC IL-8 autocrine expression. The expression of IL-8, in-turn, augments tumor angiogenesis through IL-8 Receptors on vascular endothelial cells (Figure 3). IL-1 has been demonstrated in bone resorption and IL-1 has been shown to be expressed by HBC cells, however a major gap exists in our understanding of processes that occur in HBC-Induced Osteolysis and the role of tumor-cell derived IL-1.

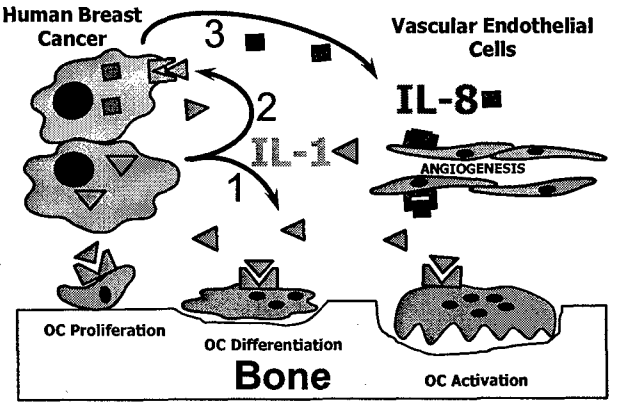

Figure 3. Hypothesis of the role of proinflammatory cytokines (IL-1 & IL-8) in human breast cancer-induced osteolysis based on our studies. 1. IL-1 can activate osteoclastogenesis, promote osteoclast (OC) activation and osteolysis *via* paracrine induction of IL-1Receptors on osteoclasts. 2. IL-1 can promote tumor progression by autocrine induction and subsequent activation of IL-8. 3. IL-8 expressed by HBC cells can support tumor progression by stimulating angiogenesis through IL-8Receptor expression on vascular endothelial cells.

E1. "SO WHAT".

1. This work demonstrates a previously unrecognized pathway (IL-1) in breast cancer-induced osteolysis, which is distinct and independent of the PTHrP model as described above in figure 2.
2. This work supports our hypothesis as described in figure 3 and suggests direct activation of osteoclasts by breast cancer derived IL-1.
2. This work is based on human samples and has direct relevance to human pathophysiology.
3. The PTHrP model is primary derived from animal and tissue culture studies. Our studies suggest that the currently used animal models of breast cancer osseous metastatic pathology my not be a true representation of the human disease.
4. Human breast cancer bone metastasis occurs in over 80% of patients with advanced breast cancer. Patients diagnosed with osseous-metastatic disease are considered terminal. Over the last few years the prognosis for patients with HBC bone metastasis has nearly doubled from less that a year to 18-24 months. As we are better able to treat patients, we must also hold out hope to those with advanced disease. With bone metastasis there is a significant decrease in the quality of life due to severe pain and complications associated with pathological fracture. Current palliative treatment consists of using bisphophonates, which decrease osteolysis, but has little effect on tumor and disease progression in the osseous metastatic lesion. Based on our work in orthopaedic oncology using human tissue, we developed the hypotheses that HBC-derived IL-1 mediates osteolysis by differentiation and activation of osteoclasts through the IL-1 Receptor. With this work, we have identified a new network of cytokines and receptors that contribute to disease progression. Identification of this pathway is the first step in developing new therapeutic.

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8. Pantschenko, A.G., et al., *The interleukin-1 family of cytokines and receptors in human breast cancer: implications for tumor progression.* Int J Oncol, 2003. **23**(2): p. 269-84.

G. APPENDICIES:

1. **A.G. Pantschenko,** R. Naujoks, R. H. Quinn, M. Sanders, G. Gronowicz. Cellular Cooperativity *via* Proinflammatory Cytokine Networks in the Human Breast Carcinoma Bone Metastatic Microenvironment. Skeletal Complications of Malignancy IV. April 28-30, 2005 Natcher Conference Center, NIH, Bethesda, MD (Attachment 1).
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ATTACHMENT 1.

Cellular Cooperativity *via* Proinflammatory Cytokine Networks in the Human Breast Carcinoma Bone Metastatic Microenvironment.

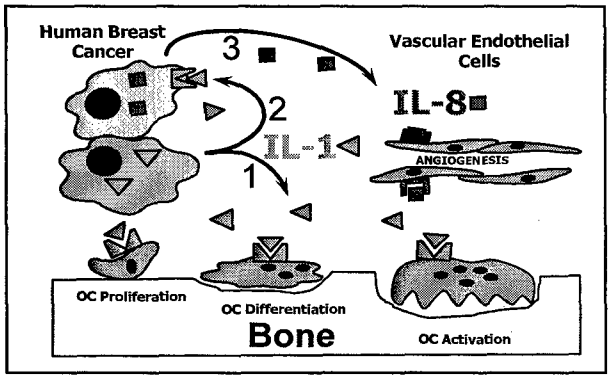
A. G. Pantschenko[1], R. Naujoks[1], R. H. Quinn[2], M. Sanders[3], G. Gronowicz[1].
[1]Dept. of Orthopaedic Surgery, University of Connecticut Health Center, Farmington, CT, USA, [2]Dept. of Orthopaedics, University of New Mexico Health Sciences Center, Albuqerque, NM, USA, [3]Dept. of Pathology, University of Connecticut Health Center, Farmington, CT, USA. E-mail: alexgp@nso2.uchc.edu

Bone metastasis in human breast carcinoma (HBC) occurs in 83% of patients with advanced disease. Breast cancer has a predilection for spreading to the bone and bone metastasis is a major cause of increased pain, morbidity and mortality. Clinically, bone metastasis causes degeneration of the bone matrix (osteolysis), hypercalcemia, pathologic fracture, and nerve-compression syndrome. The pathophysiology of human breast carcinoma-induced osteolysis (HBC-IO) involves an increase in the number and activity of osteoclasts within the HBC metastatic lesion. These observations strongly support the involvement of cell-to-cell interactions and cytokine networks. We have recently demonstrated that the expression of the proinflammatory IL-1 family of cytokines and receptors correlates with disease severity and induction of the pro-angiogenic and mitogenic cytokine, IL-8, in HBC primary tumor. Furthermore, IL-8, a product of HBC IL-1 stimulation, has recently been shown to have a greater correlation with HBC bone metastatic potential than PTHrP in the nude mouse. Since IL-1 expression has been correlated with HBC aggressiveness and IL-1 is a known activator of osteoclasts, we examined the expression of the IL-1 family of cytokines and receptors and IL-8 in HBC-IO using archival human samples and

	IL-1β			IL-1Receptor I			IL-8		
	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)	Number of (+) Samples	Percent (+) Cells	Staining Intensity (0-3)
Tumor Cells	13/16 (81%)	82%	2.3	13/16 (81%)	62%	2.0	14/17 (82%)	34%	1.3
Osteoclasts	0/16 (0%)	0%	0	15/16 (94%)	74%	2.8	0/17 (0%)	0%	0

Table 1. Immunohistochemical expression of the IL-1 family and IL-8 in HBC osteolytic lesions. No expression of IL-1RII, or IL-1Ra was observed.

immunohistochemistry. Histologic sections from pathological fracture resection or biopsy of HBC metastasis to bone from patients (mean age, 52yrs; age range, 34-83yrs; no prior radiation to site) were analyzed. We observed IL-1 and IL-8 expression by HBC cells and IL-1Receptor I expression on osteoclasts (Table 1). These data suggest that HBC-derived IL-1 is an important mediator of human breast cancer-induced osteolysis and supports our hypothesis: *1.* IL-1 can activate osteoclastogenesis, promote osteoclast (OC) activation and osteolysis *via* paracrine induction of IL-1Receptors on osteoclasts. *2.* IL-1 can promote tumor progression by autocrine induction and subsequent activation of IL-8. *3.* IL-8 expressed by HBC cells can support tumor growth and progression by stimulating angiogenesis through IL-8 Receptors expressed on vascular endothelial cells. This study suggests that IL-1 may be an important mediator of HBC pathophysiology and therefore, a potential target for therapeutic intervention.



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ATTACHMENT 2.
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Interleukin-1 Expression in Human Breast Cancer Bone Metastasis: a Newly Recognized Pathway in Breast Cancer-induced Osteolysis

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Bone metastasis in human breast carcinoma (HBC) occurs in 83% of patients with advanced disease. The pathophysiology of human breast carcinoma-induced osteolysis (HBC-IO) involves an increase in the number and activity of osteoclasts within the HBC metastatic lesion. These observations strongly support the involvement of cell-to-cell interactions and cytokine networks. We have recently demonstrated that the expression of the pro-inflammatory IL-1 family of cytokines and receptors correlates with disease severity and induction of pro-angiogenic and mitogenic cytokines (*e.g.* IL-8) in HBC primary tumor. Furthermore, IL-8, a product of HBC IL-1 stimulation, has recently been shown to have a greater correlation with HBC bone metastatic potential than PTHrP in the nude mouse.
Since IL-1 expression has been correlated with HBC aggressiveness and IL-1 is a known activator of osteoclasts, we examined the expression of the IL-1 family of cytokines and receptors in HBC-IO using archival human samples and immunohistochemistry. Samples from pathological fracture resection or biopsy of HBC metastasis from 16 patients (mean age, 52yrs; age range, 34-83yrs; no prior radiation to site; 14 samples from proximal femur) were obtained from the Dept. of Pathology, Hartford Hospital, Hartford, CT. and analyzed using the following antibodies; IL-1α, IL-1β, IL-1R1, IL-1R2, IL-8, CXC-R2, PTHrP, and anti-osteoclast antigen.
Thirteen of sixteen samples (81%) showed positive IL-1β tumor cell staining. Among these samples, the majority of tumor cells stained (82%). These thirteen samples were also positive for tumor cell staining of IL1-R1. Fifteen out of sixteen samples (94%) showed osteoclasts IL-1R1 staining. 14/16 showed positive staining of more than 50% of osteoclasts. 1/16 showed staining in 20-50% of cells and 1/16 sample showed no evidence of IL-1R1 staining of osteoclasts. This study supports the hypothesis that HBC tumor cell-induced osteolysis can be mediated through the HBC expression of IL-1 and the subsequent activation of osteoclasts via IL-1R1.

	IL-1β			**IL-1R1**		
	Number of (+) Samples	**Percent (+) Cells**	**Staining Intensity (0-3)**	**Number of (+) Samples**	**Percent (+) Cells**	**Staining Intensity (0-3)**
Tumor Cells	13/16 (81%)	82%	2.3	13/16 (81%)	62%	2.0
Osteoclasts	0/16 (0%)	0%	0	15/16 (94%)	74%	2.8

Author Disclosure Block: A.G. Pantschenko, None.

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