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TITLE: The Role of Estrogen Receptor-α in Breast Cancer Metastases to Bone

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Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)

Breast cancer osteolysis is common and the morbidity is devastating. The consequences of intractable bone pain, fracture, hypercalcemia and nerve compression syndromes are debilitating and the tumor is incurable once it has metastasized to bone. Women with bone metastases live many years with this incurable complication and are at high risk for morbidity. A more aggressive approach to prevent and treat bone metastases is a necessary addition to the standard armamentarium for breast cancer therapy in order to impact on this morbidity. Although bisphosphonates are now FDA-approved for treatment of established bone metastases and have had significant impact on bone pain and fracture, considerable advances are necessary for the eventual prevention or reversal of bone metastases. These data indicate a role for TGFβ, to potentiate ER-α-mediated transcription induced by a constitutively active ER-α. The above in vitro studies provide rationale for targeting the downstream effects of TGFβ on breast cancer cells to treat and eventually prevent osteolysis. However, in vivo, expression of wild-type ER-α and mutants Ser47Thr, Lys531Glu, and Tyr537Asn had no effect on bone metastases in a mouse model. Thus, further experiments to test the in vivo relevance of these in vitro findings are warranted.
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INTRODUCTION
Breast cancer commonly metastasizes to the skeleton in patients with advanced disease to cause either bone destruction (osteolytic metastases) or new bone formation (osteoblastic metastases) and significant morbidity\(^3\,\,^5\). Since patients with breast cancer may survive several years with their bone metastases, it is important to understand the pathophysiology of this process in order to improve therapy and prevention. The proposed work seeks to investigate tumor cell-bone interactions in breast cancer metastases to bone with specific attention to the role of 1) estrogen receptor-\(\alpha\) (ER-\(\alpha\)) in mediating tumor production of bone-active factors to cause osteolytic and osteoblastic metastases using a mouse model of bone metastases and 2) bone-derived transforming growth factor, (TGF\(\beta\)) in modulating the effects of ER-\(\alpha\) on tumor cell growth in bone. A constitutively active ER-\(\alpha\) (Tyr537Asn), identified from a bone metastases\(^1\,\,^9\), when expressed in human breast cancer cells is associated with increased production of parathyroid hormone-related protein (PTHrP), a stimulator of osteolytic metastases. Furthermore, TGF\(\beta\) enhances the ER-\(\alpha\) mediated transcriptional activity induced by the Tyr537Asn in human breast cancer cells. Defining the mechanisms responsible for breast cancer metastases to bone will provide insight into future therapy and prevention. The following hypotheses will be tested:

1. Estrogen stimulates breast cancer cell production of factors which disrupt normal bone remodeling to result in osteolytic or osteoblastic metastases.
2. Estrogen stimulates PTHrP production by TGF\(\beta\)-responsive breast cancer cells to result in osteolytic metastases. TGF\(\beta\) enhances ER-\(\alpha\) mediated transcriptional activity in breast cancer cells to stimulate growth.
3. Estrogen stimulates production of osteoblastic factors, such as ET-1, by breast cancer cells which are TGF\(\beta\) unresponsive. Restoration of TGF\(\beta\) responsiveness should result in PTHrP production and osteolytic metastases.

The following specific aims are proposed to test the hypotheses:

1. To determine the role of ER-\(\alpha\) in osteolytic or osteoblastic breast cancer metastases to bone using an in vivo model. Wild-type ER-\(\alpha\) and various mutants (Ser47Thr, Lys531Glu, and Tyr537Asn) will be stably transfected into breast cancer cell lines which are known to cause either osteolytic or osteoblastic metastases in a mouse model (MDA-MB-231, ZR-75-1, MCF-7, T47D, MDA-MB-468) as well as into cell lines which are tumorigenic in nude mice but do not cause bone metastases and clonal lines isolated. In vitro growth, PTHrP production, ET-1 production, TGF\(\beta\) responsiveness, ER-\(\alpha\) mediated transcriptional activity and effect of exogenous estrogens and antiestrogens will be tested in stable cell lines. In vivo, the effect of expression of wt ER-\(\alpha\) or mutants on bone metastases will be studied in a mouse model.

2. To determine if the effect of TGF\(\beta\) to increase ER-\(\alpha\) mediated transcriptional activity is specific for the constitutively active ER-\(\alpha\) Tyr537Asn mutant compared with wt ER-\(\alpha\) or is cell-specific.

   Stable MDA-MB-231 cell lines expressing ER-\(\alpha\) mutants or wt will be treated with TGF\(\beta\), with or without estrogens or antiestrogens. ER-\(\alpha\) transcriptional activity will be assessed by transient transfection with ERE-luc and PTHrP secretion into conditioned media will be assessed by immunoradiometric assay. ER-\(\alpha\) mutants (Ser47Thr, Lys531Glu, and Tyr537Asn) or wt will be stably expressed in ER-\(\alpha\) positive cell lines (ZR-75-1, MCF-7 (both lines), and T47D) and assessed as in the MDA-MB-231 stable constructs.

3. To determine the relationship between TGF\(\beta\) signaling and ER-\(\alpha\) mediated transcription.
Specific molecular aspects to be addressed include 1) whether these effects are mediated through the known TGF\(\beta\) serine-threonine kinase-Smad signaling pathway and 2) whether TGF\(\beta\) enhances production of nuclear receptor coactivators of ER-\(\alpha\) response, such as AIB1 or SRC-1 to enhance
ER-α dependent transcription. ER-α mutants (Ser47Thr, Lys531Glu, and Tyr537Asn) or wt will be transiently transfected into stable MDA-MB-231 clones which stably express one the following components of the TGFβ receptor-signaling pathway: truncated (dominant-negative) type II receptor, constitutively active type I receptor, Smad2 dominant-negative along with ERE-luciferase reporter construct. Cells will be treated with TGFβ and ER-α mediated transcriptional activity will be assessed. Western blots will be performed on cell lysates for nuclear coactivators of ER-α response, AIB1 and SRC-1. In each specific aim, ER-α positive (MCF-7) and ER-α negative (MDA-MB-468) cell lines in which both TGFβ responsive and -unresponsive sublines exist will be used to assess the interaction of TGFβ-ER-α within the same cell line.

**BODY**

The research accomplishments completed during year 1 are described according to the approved statement of work. Tasks 1-3 were originally scheduled for completion by month 18.

**STATEMENT OF WORK**

1. **To determine the role of ER-α in breast cancer cells which cause osteolytic or osteoblastic metastases.** (Months 1-18). Rationale: Women with ER-α positive primary tumors are more likely to develop bone metastases. Although scant data suggest that estrogen may regulate PTHrP expression in the uterus, and in a breast cancer cell line, there is no clear relationship between PTHrP and ER-α in primary breast cancer. The sparse clinical data available on ER-α expression in breast cancer bone metastases indicate that 60-75% are ER-α negative despite the fact that women with ER-α positive primary tumors are more likely to develop bone metastases. Furthermore, bone metastases were frequently ER-α negative in those patients in whom the primary tumors were ER-α positive. Recently, 3 missense mutations were identified in the ER-α gene from metastatic breast cancer: Ser47Thr, Lys531Glu, and Tyr537Asn. The first 2 ER-α mutants had similar activity to wild-type (wt) ER while the Tyr537Asn ER mutant demonstrated a potent, estradiol-independent transcriptional activity as compared to wt ER-α. This constitutive activity of Tyr537Asn was unaffected by estradiol, tamoxifen or the pure antiestrogen ICI 164,384. This Tyr537Asn mutant was derived from a bone metastases which was ER-α negative by ligand binding analysis. The mutation is located in exon 8 of the carboxy-terminal portion of the hormone-binding domain of the ER-α, a potential phosphorylation site implicated in hormone binding, dimerization, and hormone-dependent transcriptional activity. Such a mutation may be responsible for the development and progression of breast cancer metastases to bone, and since it does not bind ligand, may be classified as an ER-α negative tumor. Since bone metastases are infrequently sampled, the prevalence of this ER-α mutation is unknown. However, the exact mutation has also been identified in an endometrial carcinoma. To determine the role of ER-α in the development and progression of osteolytic metastases, we proposed to express these wild-type ER-α or mutants, Ser47Thr, Lys531Glu, and Tyr537Asn, into the ER-α negative breast cancer cell line, MDA-MB-231 which causes osteolytic bone metastases in a mouse model 1.

**Task 1:** Wild-type ER-α and various mutants (Ser47Thr, Lys531Glu, and Tyr537Asn) will be stably transfected into breast cancer cell lines which are known to cause either osteolytic or osteoblastic metastases in a mouse model (MDA-MB-231, ZR-75-1, MCF-7, T47D, MDA-MB-468) as well as into cell lines which are tumorigenic in nude mice but do not cause bone metastases and clonal lines isolated.

Stable MDA-MB-231 cell lines were constructed which express wild-type ER-α and mutants Ser47Thr, Lys531Glu, and Tyr537Asn. Over 50 clones of each different ER-α transfectants were
screened by measuring luciferase activity in the presence or absence of 17-estradiol after transient transfection with the estrogen response element linked to luciferase (ERE-luc). Among the wild-type ER-α and ER-α mutants Ser47Thr and Lys531Glu transfectants, at least 5 clones responded to 17-estradiol with a significant increase in ERE-luciferase activity. Six clones were identified from the ER-α mutant Tyr537Asn group which had increased ERE-luciferase activity in the absence of 17-estradiol. These six clones did not respond further to 17-estradiol or the antiestrogen tamoxifen. The stable cell lines were tested in a mouse bone metastases assay. The results are described below under task 3.

We have been unsuccessful in constructing stable cell lines of the other human breast cancer lines, ZR-75-1, MCF-7, T47D and MDA-MB-468 which express wild-type ER-α or ER-α mutants Ser47Thr, Lys531Glu, and Tyr537Asn. In fact, we have been unable to stably express any cDNA in ZR-75-1 and T47D, despite using multiple conditions and transfection methods. MCF-7 cells initially expressed the transfected ER-α constructs, however, clones did not remain stable. Finally, we have successfully constructed clones of MDA-MB-468 which express Smad 4 and we are in the process of testing stability of these clones.

Task 2: In vitro growth, PTHrP production, ET-1 production, TGFβ-responsiveness, ER-α mediated transcriptional activity and effect of exogeneous estrogens and antiestrogens will be tested in stable cell lines.

Figure 1 shows that stable MDA-MB-231 clones which express ER-α, (Tyr537Asn) mutant demonstrates increased ER-α mediated transcriptional activity in the absence of estradiol, as assessed by transient transfection with the ERE-luciferase compared with empty vector control. Transcriptional activity of the stable clones was not affected by estradiol treatment, but exogenous TGFβ1 increased ERE-luciferase activity in all stable clones (FIGURE 1). Furthermore, basal as well as TGFβ-stimulated PTHrP secretion by the Tyr537Asn ER mutant clones was increased compared with the empty vector controls (FIGURE 2). These data suggest that ER-α mediated transcription is associated with increased tumor production of PTHrP. This, in combination with the effect of TGFβ to enhance ER-α mediated transcription, and potentially growth, may be a mechanism for the propensity of breast cancer to metastasize to the skeleton.

FIGURE 1: TGFβ enhances ER-α mediated transcriptional activity in stable MDA-MB-231 clones which express the constitutively active ER-α [MDA/ER(Tyr537Asn)]. MDA/ER(Tyr537Asn) and empty vector control clones (MDA/pcDNA3) were transiently transfected with ERE-luciferase reporter, switched to phenol red-free media with charcoal-stripped serum at 8 hr and incubated for 24 hrs more and treated with TGFβ, estradiol or both for 24 hrs. Values represent the mean ± SEM of triplicate measurements. Statistical analysis by ANOVA.
FIGURE 2: TGFβ enhances PTHrP production by MDA-MB-231 cells which express the constitutively active ER-α [MDA/ER(Tyr537Asn)] PTHrP secretion into 24 hour conditioned media obtained from samples illustrated in figure 1. PTHrP was measured by immunoradiometric assay and corrected for cell number. Values represent the mean ± SEM of triplicate measurements. Statistical analysis by ANOVA. MDA/EV=empty vector pcDNA3 clones.

To determine if the effects of TGFβ on ER-α mediated transcription were specific to the ER-α (Tyr537Asn) mutant, we constructed stable MDA-MB-231 cell lines which expressed wild-type ER-α, or ER-α mutants which were identified in soft tissue metastases, Ser47Thr and Lys531Glu. These data, (FIGURES 3a-c) illustrate that although ER-α mediated transcription was increased in response to 17-estradiol in clones which expressed wild-type or Ser47Thr and Lys531Glu mutants, there was no additional effect of TGFβ. Furthermore, the combination of 17-estradiol and TGFβ did not increase PTHrP production over TGFβ alone. There was no significant difference between wild-type ER-α and the Ser47Thr or Lys531Glu mutants. These data suggest that the ER-α (Tyr537Asn) mutant, isolated from a bone metastasis, may confer specific properties to the breast cancer cells which facilitate osteolytic bone metastases.

FIGURE 3: Estradiol, but not TGFβ, increase ER-α mediated transcriptional activity (left panel) and PTHrP production (right panel) in stable MDA-MB-231 clones expressing the wild-type ER-α (a) or mutants Ser47Thr (b) and Lys531Glu (c) compared with empty vector control clones (MDA/pcDNA3). Clones were treated with TGFβ, estradiol or both. ERE luciferase activity and PTHrP measurements were assessed as in figure 7 & 8. Values represent the mean ± SEM of triplicate measurements. Statistical analysis by ANOVA.
Task 3: In vivo, the effect of expression of wt ER-α or mutants on bone metastases will be studied in a mouse model. Stable MDA-MB-231 clones from figures 1-3 were inoculated into nude mice to determine the effect of wild-type ER-α or ER-α mutants Ser47Thr, Lys531Glu, and Tyr537Asn on bone and soft tissue metastases.

Figure 4: Effect of ER-α, wild-type and mutants, on radiographic bone metastases by MDA-MB-231. Lesion number and lesion area were quantified by computerized image analysis. There was no significant difference in osteolytic lesion number or osteolytic lesion area between MDA-MB-231 clones which express the empty vector, wild-type or ER-α mutants.

**Survival curve**

Figure 5: Effect of ER-α, wild-type and mutants, on survival of mice bearing bone metastases by MDA-MB-231. Survival was similar in all groups.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of stable MDA-MB-231 cell lines which express wild-type ER-α and mutants Ser47Thr, Lys531Glu, and Tyr537Asn.
- Determination that TGFβ increased PTHrP production and ER-α mediated transcription in stable MDA-MB-231 clones which expressed the constitutively active ER-α mutant Tyr537Asn.
- Expression of wild-type ER-α and mutants Ser47Thr, Lys531Glu, and Tyr537Asn by MDA-MB-231 breast cancer had no effect on the development and progression of bone metastases in a mouse model.

REPORTABLE OUTCOMES

*Manuscripts, abstracts, presentations:*
The following were supported by this Academic Award:

**Manuscripts**

2. Guise TA. From chondrocytes to cancer: Fibroblast growth factor receptor 3 (FGFR3). Commentary in IBMS BoneKEy 2001 Mar 7 10.1138/ibmske;2001016
12. Guise TA. How Metastases Home to Bone: The Attraction of Chemokines. IBMS BoneKEy 2002 Jul 9 0.1138/ibmske;2002052

**Presentations**

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7. Role of PTHrP in malignancy. Medicine Grand Rounds, Henry Ford Hospital, Detroit, MI, May, 2000


22. Molecular Mechanisms of Bone Metastases: Implications for Therapy. Department of


34. Biology of metastasis in bone injury specific to breast cancer. Carroll W. Feist Symposium; Louisiana State University Health Science Center Shreveport, LA. May 2002.

Abstracts


**Patents and licenses applied for or issued:** None

**Degrees obtained that are supported by this award:** None

Development of cell lines, tissue or serum repositories: Stable cell MDA-MB-231 cell lines which express wild-type ER-α and mutants Ser47Thr, Lys531Glu, and Tyr537Asn.

**Informatics such as data bases and animal models:** None

**Funding obtained based on work supported by this award:**

1. National Institutes of Health (NCI), “Breast cancer osteolysis: PTHrP regulation by TGFβ”. (R01-CA69158; Guise, PI, 25% effort). Total costs:

**Employment or research opportunities applied for and/or received on training supported by this award:** None

**CONCLUSIONS**

Breast cancer osteolysis is common and the morbidity is devastating. Not only are the consequences of intractable bone pain, fracture, hypercalcemia and nerve compression syndromes debilitating, but the tumor is incurable once it has metastasized to bone. The fact remains that women with breast cancer and bone metastases live many years with this incurable complication and, thus, are at high risk for such morbidity. A more aggressive approach to prevent the development of bone metastases as well as to treat established lesions is a necessary addition to the standard armamentarium for breast cancer therapy in order to impact on this morbidity. Although bisphosphonates are now FDA-approved for treatment of established bone metastases and have had significant impact on bone pain and fracture, considerable advances are necessary for the eventual prevention or reversal of bone metastases. These data indicate a central role for TGFβ to potentiate ER-α-mediated transcription induced by a constitutively active ER-α. The above in vitro studies provide rationale for targeting the downstream effects of TGFβ on breast cancer cells to treat and eventually prevent osteolysis. However, in vivo, expression of wild-type ER-α and mutants Ser47Thr, Lys531Glu, and Tyr537Asn
had no effect on the development and progression of bone metastases in a mouse model. Thus, further experiments to test the in vivo relevance of these in vitro findings are warranted.
REFERENCES