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

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13. ABSTRACT (Maximum 200 Words) Breast cancer commonly metastasizes to the skeleton in patients with advanced disease to cause either bone destruction or new bone formation. 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-α (ER-α) 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 β (TGFβ) in modulating the effects of ER-α on tumor cell growth in bone. 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 TGFβ-responsive breast cancer cells to result in osteolytic metastases. TGFβ enhances ER-α-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 TGFβ unresponsive. Restoration of TGFβ responsiveness should result in PTHrP production and osteolytic metastases. Three specific aims were proposed to test the hypotheses and we report here the progress for Specific aim 1 in year 1 of this Academic Award: 1. To determine the role of ER-α in osteolytic or osteoblastic breast cancer metastases to bone using an in vivo model. Stable MDA-MB-231 cell lines were constructed which express wild-type ER-α and mutants Ser47Thr, Lys531Glu, and Tyr537Asn. MDA-MB-231 clones which express ER-α(Tyr537Asn) mutant demonstrated increased ER-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. Basal as well as TGFβ-stimulated PTHrP secretion by the Tyr537Asn ERα mutant clones was increased compared with the empty vector controls. 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.
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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. 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-α (ER-α) 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 β (TGFβ) in modulating the effects of ER-α on tumor cell growth in bone. A constitutively active ER-α (Tyr537Asn), identified from a bone metastases, when expressed in human breast cancer cells is associated with increased production of parathyroid hormone-related protein (PTHrP), a stimulator of osteolytic metastases. Furthermore, TGFβ enhances the ER-α-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 TGFβ-responsive breast cancer cells to result in osteolytic metastases. TGFβ enhances ER-α-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 TGFβ unresponsive. Restoration of TGFβ 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-α in osteolytic or osteoblastic breast cancer metastases to bone using an in vivo model. 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. 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. In vivo, the effect of expression of wt ER-α or mutants on bone metastases will be studied in a mouse model.

2. To determine if the effect of TGFβ to increase ER-α-mediated transcriptional activity is specific for the constitutively active ER-α Tyr537Asn mutant compared with wt ER-α or is cell-specific.

Stable MDA-MB-231 cell lines expressing ER-α mutants or wt will be treated with TGFβ, with or without estrogens or antiestrogens. ER-α-transcriptional activity will be assessed by transient transfection with ERE-luc and PTHrP secretion into conditioned media will be assessed by immunoradiometric assay. ER-α mutants (Ser47Thr, Lys531Glu, and Tyr537Asn) or wt will be stably expressed in ER-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 TGFβ signaling and ER-α-mediated transcription.

Specific molecular aspects to be addressed include 1) whether these effects are mediated through the known TGFβ serine-threonine kinase-Smad signaling pathway and 2) whether TGFβ enhances production of nuclear receptor coactivators of ER response, such as AIB1 or SRC-1 to enhance ER-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 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-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 in primary breast cancer. The sparse clinical data available on ER expression in breast cancer bone metastases indicate that 60-75% are ER-negative despite the fact that women with ER-positive primary tumors are more likely to develop bone metastases. Furthermore, bone metastases were frequently ER-negative in those patients in whom the primary tumors were ER-positive. Recently, 3 missense mutations were identified in the ER-α gene from metastatic breast cancer: Ser47Thr, Lys531Glu, and Tyr537Asn. The first 2 ER 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. 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-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-negative tumor. Since bone metastases are infrequently sampled, the prevalence of this ER 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.

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 17β-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 17β-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 17β-estradiol. These six clones did not respond further to 17β-estradiol or the antiestrogen tamoxifen.

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 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-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 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-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-mediated transcription was increased in response to 17β-estradiol in clones which expressed wild-type or Ser47Thr and Lys531Glu mutants, there was no additional effect of TGFβ. Furthermore, the combination of 17β-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 have been 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. These experiments are in progress.
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-mediated transcription in stable MDA-MB-231 clones which expressed the constitutively active ER-α mutant Tyr537Asn.

REPORTABLE OUTCOMES

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

Presentations

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 applied for based on work supported by this award:
2. Burroughs Wellcome Fund Clinical Scientist Award in Translational Research, “Endothelin A Receptor Blockade: Consequences for Bone Physiology in Normal & Pathologic Conditions” (1002256; Guise, PI, 25% effort).

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 studies provide rationale for targeting the downstream effects of TGFβ on breast cancer cells to treat and eventually prevent osteolysis.
REFERENCES
Expression of a Constitutively Active Estrogen Receptor (ER)-α Increases Parathyroid Hormone-Related Protein (PTHrP) Production And Transforming Growth Factor (TGF)-β-Responsiveness by Human Breast Cancer Cells. JJ Yin, JM Chirgwin, SAW Fuqua, TA Guise. University of Texas Health Science Center, San Antonio

Breast cancer patients with ER-positive primary breast cancers are more likely to develop bone metastases, but the molecular mechanisms responsible for these clinical observations are unknown. There has been an increase in the recent understanding of mediators involved in breast cancer osteolysis. PTHrP mediates breast cancer osteolysis and its expression is increased in breast cancer metastases to bone, likely a result of bone-derived TGFβ. Although estrogen has been shown to increase PTHrP expression in the uterus as well as in a breast cancer cell line, no clear relationship between PTHrP and ER in primary breast cancer has been demonstrated. To understand the relationship between estrogen receptor and osteolysis, we determined if a missense mutation in ER-α, which was detected in a human bone metastases, had the capacity to alter tumor cell production of PTHrP, in the basal state and in response to TGFβ. The mutant, in which tyrosine at amino acid 537 is replaced with asparagine, Tyr537Asn, demonstrated a potent, estradiol-independent transcriptional activity as compared to wild-type (wt) ER. The point mutation is located in exon 8 of the carboxy-terminal portion of the hormone-binding domain of the ER, to which dimerization and transcriptional functions have been ascribed. It is also a potential phosphorylation site implicated in hormone binding, dimerization, and hormone-dependent transcriptional activity. This constitutive activity of Tyr537Asn was unaffected by estradiol, tamoxifen or the pure antiestrogen ICI 164,384. The cDNA for this Tyr537Asn ER mutant was transfected into MDA-MB-231 human breast cancer cells, which lack ER-α, and 4 single clones were isolated which demonstrated increased ER-mediated transcriptional activity in the absence of estradiol, as assessed by transient transfection with the estrogen response element linked to luciferase (ERE-luc) compared with 2 empty vector control clones. Receptor expression in stable clones was demonstrated by western blot. The transcriptional activity of the stable clones was not affected by estradiol treatment, but treatment with exogenous TGFβ1 resulted in a significant increase in ERE-luciferase activity in all stable clones compared with empty vector controls. Furthermore, basal as well as TGFβ-stimulated PTHrP secretion by the Tyr537Asn ER mutant clones was significantly increased compared with the empty vector controls. Growth rates in vitro did not differ significantly between the Tyr537Asn mutant clones and the empty vector in the basal state or in response to exogenous 17β-estradiol. However, Tyr537Asn mutant clones were slightly, but significantly growth inhibited by TGFβ. These data suggest that ER-α-mediated transcription is associated with increased tumor production of PTHrP, basally and in response to TGFβ. Furthermore, the data suggest convergence between the estrogen and TGFβ signaling pathways. The combined effect of the Tyr537Asn mutant to enhance tumor-produced PTHrP and TGFβ to enhance ER-α-mediated transcription may be a mechanism for the propensity of breast cancer to metastasize to the skeleton.