Inhibition of Skeletal Metastasis by Ectopic ERα Expression in ERα-Negative Human Breast Cancer Cell Lines

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Abstract

Some hormone-independent breast cancers lack functional estrogen receptors (ERs) and show evidence of a more aggressive metastatic phenotype. A protective role of the ER has also been suggested in hormone-resistant breast cancer progression. In this study, we have investigated the effect of the ectopic expression of human ERα on the bone-metastatic potential of highly metastatic ERα-negative human breast cancer MDA-MB-231 and MDA-MB-435-F-L cell lines in an experimental model of bone metastasis in nude mice. ERα overexpression had no effect on the growth of both cell lines but reduced the expression of integrin αvβ3 and the receptor activator of NF-κB, which are known to promote bone metastasis. A significant reduction in the incidence of osteolytic bone metastasis was observed by X-ray imaging of the legs and arms of mice inoculated with ERα-expressing clones of MDA-MB-231 cells in comparison to controls. Ectopic expression of ERα in MDA-MB-435-F-L cells also reduced their widespread skeletal metastasis to the legs, arms, spine, and mandible, as detected by whole-mouse enhanced green fluorescent protein imaging. Our study indicates for the first time that these ERα-negative breast cancer cells can inhibit their aggressive bone-metastatic potential in an experimental bone metastasis model.

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Introduction

In the United States, breast cancer is the most common malignancy and second leading cause of cancer-related deaths in women [1]. Metastasis to distant organs, especially to the bone and lungs, is the leading cause of morbidity and death in breast cancer patients. Estrogen, which exerts its effects by binding to estrogen receptor α (ERα; expressed in about 70% of breast cancers) and ERβ, has been implicated in the malignant progression of breast cancer [2]. As tumor progresses, ERα-positive tumors may lose ERα expression and become estrogen-independent with increasing risk of metastasis [3]. These invasive and metastatic ERα-negative tumors respond poorly to endocrine therapy, chemotherapy, and radiation treatment [4,5]. Therefore, the probability of death is much greater for patients with ERα-negative breast tumors than for patients with ERα-positive breast tumors [6]. However, the presence of ERα in tumors has been related to a favorable response to endocrine therapy and improves overall survival [7]. In a recent report, epigenetically reactivated ERα in ER-negative breast cancer cells restored their sensitivity to endocrine therapy [8]. In advance breast cancer, bone metastasis is prevalent in > 80% of patients (http://www.cancer.org/cancerinfo). In addition to debilitating osteolysis, tumor cells in the bone marrow correlated with recurrence and poor patient survival [9]. A clinical study showed that patients with ERα/progesterone (PR+) tumors had a lower frequency of disseminated tumor cells in the bone marrow, compared to patients with ERα /PR− tumors [10]. In this study, we have stably transfected two highly metastatic ERα-negative human breast cancer cell lines, MDA-MB-435-F-L and MDA-MB-231, with ERα and have evaluated their bone-metastatic potential in comparison to control cells in an intracardiac injection model of skeletal metastasis in nude mice. Our results showed for the first time that these ERα-negative cell lines expressing functional ERα significantly inhibited both tumor cell homing to the skeleton and osteolysis, indicating that restoration of ERα might be a potential therapeutic approach for the inhibition of bone metastasis in hormone-independent breast cancer.

Materials and Methods

Animals and Cell Lines

Four- to 5-week-old female athymic nude mice (obtained from Harlan Sprague Dawley, Inc., Indianapolis, IN) were used...
for in vivo animal experiments. The animals were housed under specific pathogen-free conditions. All animal protocols were approved and monitored by the institutional animal care and use committee.

MDA-MB-435-F-L cells, which are highly invasive and metastatic variants of human breast carcinoma MDA-MB-435 cells, were isolated in our laboratory [11]. The human breast cancer cell line MDA-MB-231 was originally obtained from the American Type Culture Collection (Manassas, VA). These cell lines were cultured in McCoy’s 5A medium supplemented with pyruvate, vitamins, amino acids, antibiotics, and 10% fetal bovine serum, as previously described [12]. To detect their potential for early metastasis to the bone, we stably transfected the enhanced green fluorescent protein (EGFP) expression plasmid pEGFP-N1 (Clontech Laboratories, Inc., Mountain View, CA) into these cell lines and sorted out a pool of EGFP-expressing cells for further studies.

Transfection and Expression of ERα in Human Breast Cancer Cell Lines

MDA-MB-231 and MDA-MB-435-F-L cells expressing EGFP were transfected with a retroviral ERα expression vector, ERα/pBabe-Puro, or pBabe-puro as control. Stable transfectants resistant to puromycin treatment (3.0 μg/ml) were isolated by limiting dilution clones. Puromycin-resistant control clones were pooled as a control cell line. To confirm whether ectopic ERα is functional, both MDA-MB-231 and MDA-MB-435-F-L control cells and their respective ERα clones were transiently cotransfected with an estrogen-responsive promoter luciferase plasmid (thymidine kinase–ERE–Luciferase) and a β-galactosidase (β-gal) expression plasmid. At 3 hours after transfection, cells were treated with or without 17β-estradiol (E2; 10−7 M) for 20 hours. Luciferase activity normalized with β-gal activity in cell lysates was then determined as described previously [13].

Cell Proliferation Assay

MDA-MB-231 and MDA-MB-435-F-L control cells and their respective ERα clones were plated in a 96-well plate at 2000 cells/well, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to obtain the relative cell number after 5 days of incubation, as described previously [14].

Western Blot Analysis

The cell lysates of both control cells and ERα clones of MDA-MB-231 and MDA-MB-435-F-L cells were used in Western blot analysis, as described previously [15]. Antibody to integrin αv was obtained from Cell Signaling Technology (Beverly, MA), and antibodies to ERα, integrin αv, and the receptor activator of NF-κB (RANK) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Experimental In Vivo Bone and Lung Metastasis Assay

An intracardiac injection model for experimental bone metastasis was used for this study, as previously described [16]. Briefly, MDA-MB-231 and MDA-MB-435-F-L control cells and their respective ERα clones were harvested from subconfluent exponentially growing cultures. The cells were injected into the left cardiac ventricle of anesthetized female nude mice (5 weeks old) with a 27-gauge needle attached to a 1-ml syringe using a micromanipulator. Each mouse was injected with 106 cells in 0.1 ml of phosphate-buffered saline, and successful injections were indicated by the pumping of red blood into the syringe. Development of bone metastasis induced by EGFP-expressing MDA-MB-435-F-L cells was monitored at regular intervals by whole-animal imaging for green fluorescence to detect EGFP-expressing tumor cells growing in the legs, arms, spine, and mandible bones, using a Nikon SMZ1500 (Nikon, Tokyo, Japan) fluorescence stereoscope attached to a CoolSNAP CCD camera (Photometrics, Tucson, AZ). EGFP expression was mostly lost in MDA-MB-231 cells after transfection with pBabe-puro or ERα/pBabe-puro. Therefore, X-ray radiographs taken with Faxitron were used for the detection of any bone lesion in mice injected with MDA-MB-231 cells. After the termination of experiment at about 4 weeks, whole lungs were excised, and EGFP-expressing metastatic cancer cell colonies were visually observed and counted under an inverted fluorescence microscope. Bone tissues were fixed in 10% neutral-buffered formalin (Fisher Scientific, Houston, TX) for 24 hours at room temperature, decalcified in 10% EDTA, and embedded in paraffin. Sections were stained with hematoxylin, eosin, orange G, and phloxine, and the presence of metastatic tumors and bone osteolysis in the femora and tibiae was examined above and below a growth plate under a microscope.

Results and Discussion

The bone is the most common site of metastases in patients with breast carcinoma. Patients with ER-positive bone metastases usually have a prognosis better than that of patients with ER-negative bone metastases [17], suggesting a protective role of ER in tumor progression associated with bone metastasis. ER-positive tumors in breast cancer patients are more differentiated and have lower metastatic potential, in comparison to ER-negative tumors [18]. In this study, we examined whether stable reintroduction of functional human ERα in ERα-negative human breast cancer cells could alter their bone-metastatic potential in an experimental bone metastasis model. We used two bone-metastatic ERα-negative human breast cancer cell lines for the stable transfection of ERα, MDA-MB-231 cells (which are highly osteolytic) [19], and MDA-MB-435-F-L cells (which are highly invasive and capable of widespread skeletal metastasis) [11] in animal models. We isolated and characterized the ERα clones of each cell line after stable transfection with an expression vector for wild-type human ERα. The expression of ERα was detected by Western blot analysis in MDA-MB-231 ERα clones 11 and 15 and in MDA-MB-435-F-L ERα clones 3 and 6, even though it was lower than that in ERα-positive breast cancer MCF-7 cells (Figure 1A). ERα was undetectable in control vector–transfected cells (Figure 1A). The estrogen sensitivity of the ERα clones of both cell lines was confirmed with E2-mediated induction of an
estrogen-responsive promoter activity reported by luciferase activity and compared also with the ERα-positive human breast cancer MCF-7 cell line. Both ERα-expressing clones of MDA-MB-231 cells (Figure 1B) and MDA-MB-435-F-L cells (Figure 1C) showed high levels of promoter activity, which was further stimulated after treatment with E2, suggesting that ectopic ERα is functional.

Although the expression of ERα greatly increased estrogen-responsive promoter activity, it showed a limited effect on the growth of both cell lines on plastic (Figure 1D). Previous studies have shown that the expression of ectopic ERα in ERα-negative human mammary epithelial cells led to growth inhibition through treatment with E2 at concentrations between 10^{-10} and 10^{-7} M [20–22]. However, we only observed a moderate growth inhibition of some ERα-transfected clones through treatment with E2 within the same concentration range (data not shown). This discrepancy is likely due, in part, to the fact that ectopic ERα levels in our clones appear to be lower than those in published studies [20–22] when the ERα level in MCF-7 cell was used as standard.

To investigate the bone-metastatic potential of ERα-expressing clones compared to that of parental cells in both cell lines, we used an intracardiac injection model of experimental metastasis. Female nude mice were inoculated with MDA-MB-231 and MDA-MB-435-F-L control cells or their respective ERα clones through the left ventricle of the heart. Bone metastasis induced by MDA-MB-435-F-L control cells and ERα clones (clones 3 and 6) was monitored by whole-mouse EGFP imaging, as described earlier [14]. Because MDA-MB-231 transfectants did not retain EGFP expression and the cell line was known to induce osteolytic bone metastasis, whole-mouse X-ray imaging was used to detect osteolytic bone metastasis induced by MDA-MB-231 control and ERα clones. Whole-mouse EGFP imaging on week 4 after inoculation revealed that the expression of ERα

Figure 1. Ectopic expression of functional ERα in human breast cancer cells. (A) MDA-MB-231 and MDA-MB-435-F-L cells were transfected with either a retroviral ERα expression vector or an empty vector, which carried a puromycin-resistant gene. Puromycin-resistant clones were selected, and the expression of ERα was determined by Western blot analysis with cell lysates from empty vector–transfected controls (Con) and ERα clones of the MDA-MB-231 and MDA-MB-435-F-L cell lines. In a separate Western blot analysis, the ERα level in MDA-MB-231 Cl.15 was compared with that in ERα-positive human breast cancer MCF-7 cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were measured in cell lysates to show equal sample loading. (B and C) To confirm the expression of a functional ERα in ERα clones of MDA-MB-231 (B) and MDA-MB-435-F-L (C) cells, the control cells, ERα clones and MCF-7 cells, were transiently cotransfected with an estrogen-responsive promoter luciferase plasmid (ERE–thymidine kinase–Luciferase) and a β-gal expression plasmid. Cells were treated with 10^{-7} M E2. Luciferase and β-gal activities in cell lysates were determined. Luciferase activity normalized with β-gal activity is presented as the mean ± SEM for each treatment from triplicate transfections. *Significant difference (P < .05) from the corresponding control without E2 treatment, by Student’s t test. (D) To determine the effect of ERα expression on the growth of MDA-MB-231 and MDA-MB-435-F-L cells, the controls and two ERα clones were plated in a 96-well plate, and MTT assay was performed to obtain the relative cell number. Each point represents the mean ± SEM from four wells. The means of the three cells at each time point were not significantly different by ANOVA.
markedly reduced the widespread skeletal metastasis incidence induced by MDA-MB-435-F-L cells (Table 1A). The incidence of metastasis in the femur/tibia, spine, and mandible of control cell-inoculated mice was 75%, whereas in ERα clone-inoculated mice, it ranged between 0% and 33%. The difference in metastasis incidence between the femur/tibia and the mandible was statistically significant by Fisher’s exact probability test. Similarly, the incidence of osteolytic bone metastasis as detected by X-ray imaging in the legs and arms of mice inoculated with ERα clones of MDA-MB-231 cells for 3 weeks was significantly reduced in comparison to that of control mice ($P < .05$) (Table 1B and Figure 2A). It was 80% and 40%, respectively, in the legs and arms of control cell-inoculated mice, whereas in ERα clone-inoculated mice, it ranged between 0% and 10%. We also examined the presence of tumor cells in stained histologic sections of the femur and tibia of mice inoculated with both control cell lines or their ERα-expressing clones. Representative bone histology pictures are shown in Figure 2, B and C. The incidences of bone metastasis in the femora and tibiae obtained by histology examination were identical to those obtained by EGFP imaging or X-ray imaging, as shown in Table 1. In these stained bone sections, it is evident that the growth of metastatic tumors was associated with osteolysis, as reflected by the loss of trabecular bones in control cell-inoculated mice (Figure 2, B and C). Because the majority of mice inoculated with ERα clones did not have tumor cells in the tibiae and femora, it is not clear whether ERα expression in the two breast cancer cell lines can also inhibit osteolysis. Thus, our studies indicate that the inhibition of skeletal metastasis by the expression of ERα in ERα-negative breast cancer cells appears due to the inhibition of cancer cell homing in the bone marrow. At present, it is not clear whether ERα cells failed to invade the bone marrow or whether they did not proliferate in the bone marrow. Ectopic expression of ERα in MDA-MB-231 cells has been previously shown to inhibit its invasive potential \textit{in vitro} and to reduce the number and burden of lung metastases when the cells were inoculated through the tail vein of nude mice [22]. Thus, it is likely that the attenuated invasive potential after ERα expression contributed significantly to the loss of bone-metastatic potential in MDA-MB-231 and MDA-MB-435-F-L cells.

Integrins, which are cell surface receptors involved in cell adhesion, migration, invasion, and survival, have been implicated in the malignant progression of cancer [23]. Several studies indicated a role for integrin $\alpha_v\beta_3$ in the promotion of both spontaneous and experimental metastases to the bone in animal models [14,24–26]. In a clinical study, integrin $\alpha_v\beta_3$ was found to be overexpressed in metastatic breast cancer cells within the bone, compared to primary breast adenocarcinoma [27]. The level of integrin $\alpha_v\beta_3$ was found to be significantly lower in tumors initiated from an ERα-transfected human endometrial cancer cell line than in tumors originated from an ERα-negative parental cell [28]. Because systemic treatment with an antagonist of integrin $\alpha_v\beta_3$ has been shown to inhibit bone metastasis induced by MDA-MB-435 parental cells [24], we examined whether integrin $\alpha_v\beta_3$ was associated with ERα-induced inhibition of bone metastasis by MDA-MB-435-F-L cells. As shown in Figure 3A, the protein levels of both integrin $\alpha_v$ and $\beta_3$
subunits were markedly lower in the two ERα clones of MDA-MB-435-F-L cells than in control cells, suggesting that the inhibition of integrin αvβ3 may be one of the mechanisms by which ERα inhibits bone metastasis. The expression of integrin αv in MDA-MB-231 cells was undetectable with Western blot analysis. Previous studies have shown that ectopic expression of integrin αvβ3 stimulated the bone-metastatic potential of MDA-MB-231 cells [26]. Thus, ERα-induced inhibition of the bone-metastatic potential of MDA-MB-231 is not likely due to the inhibition of integrin αvβ3 expression.

Another cell surface receptor that has been shown to promote the homing of cancer cells to the bone is RANK. It has been shown that cytokine RANK ligand, which is expressed by stromal cells and osteoblasts, can trigger the migration and bone metastasis of human epithelial cancer cells expressing RANK [29]. We found that both MDA-MB-231 and MDA-MB-435-F-L cells express RANK protein, which was noticeably reduced in their respective ERα clones (Figure 3, A and B). Thus, reduction of RANK expression in ERα-expressing cells may be another mechanism for the impairment of bone-metastatic potential by ERα. Future studies will be necessary for the elucidation of whether the dramatic blockade of bone-metastatic potential in the two breast cancer cell lines by the expression of ERα is mainly due to the attenuation of the integrin αvβ3 and RANK pathways.

In summary, our study demonstrates that restoration of functional ERα expression in ERα-negative human breast cancer cells can block their aggressive bone-metastatic potential, supporting the notion that ERα confers a less aggressive phenotype of breast cancer. Although the mechanism by which ERα suppresses metastatic potential is currently not clear, our data suggest a potential involvement of the integrin αvβ3 and/or the RANK pathway in mediating the metastasis-suppressive action of ERα signaling.

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References


