Expression of Receptor Activator of Nuclear Factor-κB Ligand Is Inversely Correlated with Metastatic Phenotype in Breast Carcinoma

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ABSTRACT
During normal bone remodeling, the receptor activator of nuclear factor-κB (RANK) interacts with its ligand RANKL, which is present on pre-osteoclasts, resulting in bone resorption and initiation of new bone formation. When breast cancer metastasizes to bone, normal bone remodeling is disturbed by invasion of tumor cells, resulting in osteolytic lesions. We have studied the expression of both RANK and RANKL in 10 nonneoplastic breast samples, 58 infiltrating ductal carcinoma (IDC), and 43 breast cancer bony metastases (BTM). RANK seemed to be present in all samples tested. However, whereas RANKL expression was observed in 90% of nonneoplastic breast, RANKL expression was only observed in 62% of nonmetastatic IDC, 31% of metastatic IDC, and 2% of osteolytic BTM lesions. This decreased or absent expression of RANKL in the tumor cells may allow RANK, which is normally expressed as a receptor on the cell surface, to target RANKL present on the cell surface of normal osteoblasts and stromal cells of the bone. Stimulation of the normal osteoblasts and stromal cells by the tumor cells may then lead to secondary osteoclastogenesis, resulting in the osteolytic phenotype common to breast metastases.

INTRODUCTION
Breast cancer is a major cause of death among women in United States and worldwide (1). Despite improvements in technology and early diagnosis, many patients still succumb to the disease if the primary tumor metastasizes to secondary organs such as bone. Thus, the ability to predict the metastatic behavior of a patient’s tumor and the ability to eradicate or control recurrent disseminated malignancy remain as major clinical challenges (2).

Breast cancer avidly metastasizes to bone to form osteolytic lesions, but the factors favoring breast cancer growth in bone remain to be resolved. It has been proposed that bone destruction by breast cancer is mediated either directly by tumor cells or indirectly by osteoclasts (3, 4). In 1998, osteoclast differentiation factor, also known as receptor activator of nuclear factor-κB ligand (RANKL), was identified (5). RANKL is expressed on stromal cells and osteoblasts and is thought to interact with RANK receptor on pre-osteoclasts (6). This interaction leads to osteoclastogenesis and bone resorption. Thus, RANK and RANKL seem to play key roles in bone remodeling.

Expression of RANKL in other tumor types such as prostate cancer has been reported earlier (7, 8). Studies in the mouse model systems have also generated evidence for the involvement of RANKL in metastatic bone destruction (9, 10). Several breast cancer cell lines have been reported to up-regulate RANKL expression in osteoblasts or bone marrow stromal cells and to enhance metastasis in mouse models of breast cancer (11, 12). When mouse bone marrow cells were co-cultured with the breast cancer BALB/c-MC cell line, the cancer cells induced RANKL expression in bone marrow cells and suppressed osteoprotegerin levels in the metastatic foci, resulting in pathologic osteoclastogenesis and bone destruction (11, 12). Similarly, when the human breast cancer cell line MCF-7 was transfected with the PTHrP gene and co-cultured with murine osteoblasts, an increase in RANKL mRNA levels was observed in the normal murine osteoblasts and induction of osteoclastogenesis occurred (13). These reports suggest that the tumor cells had an impact on the RANK signaling pathway in the normal osteoblasts and stroma that led to increased osteoclastogenesis. However, very little information is available on expression of RANKL in primary human breast tumors and osteolytic bone metastases. We hypothesized that expression of RANKL might correlate with metastatic phenotype in different stages of breast cancer so that RANKL might prove to be a diagnostic indicator of metastasis. To test this, we chose to study the expression of RANKL in nonneoplastic breast (NNB), infiltrating ductal carcinoma (IDC; both metastatic and nonmetastatic), and breast cancer bony metastases (BTM) tissues.

MATERIALS AND METHODS
Patient Material. Normal breast tissue and 58 primary tumors (26 nonmetastatic IDC and 32 metastatic IDC) were obtained as tissue arrays from Imgenex (San Diego, CA), whereas 43 breast cancers metastatic to bone were obtained as paraffin-embedded archival samples from the Department of Pathology,
University of Connecticut Health Center (Farmington, CT), Fox Chase Cancer Center (Philadelphia, PA), and Cooperative Human Tissue Network (Philadelphia, PA). All surgical samples were obtained from material that had been collected following informed consent in pathology archives. The samples were stripped of all identifiers and anonymously coded prior to our receipt. Samples were obtained from patients ages 32 to 85 years. The mean age for all the samples was 53.9 years.

**Immunohistochemistry of Tumors.** Slides were prepared from individual paraffin-embedded archival tumor samples, which were cut using a microtome onto polylsine-coated slides, or from prepared tumor array slides (Imgenex). They were stained as described previously (14). Briefly, slides were deparaffinized with xylene, rehydrated in alcohol, and treated with 4 N HCl at 37°C for 10 minutes to retrieve the antigen. Samples were then rinsed in distilled water and stained with RANK or anti-RANKL antibodies (Alexis, San Diego, CA) using Histostain-SP kit (Zymed, South San Francisco, CA). Samples were coverslipped for examination and then photographed using an Olympus BX-60 microscope and OpenLab image capture software (Improvision, Boston, MA). RANKL expression was scored as positive or negative expression by independent visual comparison between the samples by the three investigators and a consensus result used in the analysis. An additional tumor section was also stained with H&E for pathologic evaluation.

**Statistical Analysis.** Correlation among RANKL expression, patient age, and metastatic phenotype was done using multivariate ANOVA as well as \( \chi^2 \) and Fisher’s exact tests done using the GraphPad Instat version 3.0b for Macintosh (GraphPad Software, San Diego, CA).

**RESULTS AND DISCUSSION**

We examined the expression of RANKL in 10 NNB samples as well as 58 IDC samples and 43 BTM samples. RANKL expression was seen in the epithelial cells of 9 of the 10 (90%) NNB samples (Fig. 1A) but in the tumor cells of only 1 of the 43 (2%) BTM samples (Fig. 1D). Of the 58 IDC samples, 26 were from tumors with no metastatic involvement, whereas 32 were from tumors with metastatic involvement. Of the 26 IDC cases without metastases, 16 (61.5%) samples were positive for expression of RANKL in the epithelial cells of the tumor (Fig. 1B), whereas of the 32 IDC cases with metastatic involvement. Of the 26 IDC cases without metastases, 16 (61.5%) samples were positive for expression of RANKL in the epithelial cells of the tumor (Fig. 1C).

Multivariate ANOVA analysis correlating patient age, RANKL expression, and metastatic phenotype showed no statistical significance for patient age in our analysis (\( P = 0.2394 \)). However, analysis of the expression of RANKL in the NNB versus BTM samples did show that absence of RANKL expression in the tumor cells strongly correlated with the bone metastatic phenotype (\( P < 0.0001 \)).

Furthermore, a comparison of NNB with IDC segregated based on metastatic phenotype confirmed that there was a highly significant difference in correlation of expression of RANKL and the metastatic phenotype. Comparison of NNB with IDC tumors regardless of metastatic phenotype revealed that absence of RANKL expression in the tumor cells was correlated with IDC phenotype (\( P = 0.0136 \)). Moreover, when IDC samples were subdivided according to metastatic phenotype, comparison of NNB with IDC that had no metastatic involvement revealed no statistically significant correlation of RANKL expression in the tumor cells (\( P = 0.1274 \)), whereas a comparison of NNB with IDC that did have evidence of metastatic involvement did show a statistically significant correlation of RANKL expression in the tumor cells (\( P = 0.0023 \)). Further, comparison of IDC with respect to metastatic phenotype revealed that the absence of RANKL in
the tumor cells differentiated significantly between metastatic and nonmetastatic IDC samples \((P = 0.0334)\). This suggests that loss of RANKL expression in the tumor cells was an important indicator of metastatic phenotype.

Two models have been proposed to explain osteolytic bone metastasis. In one case, growth factors, such as transforming growth factor-\(\beta\), are secreted by tumor cells that up-regulate PTHrP in the stromal cells, which in turn raise the levels of RANKL expressed on osteoblasts, so that osteoblasts interact with pre-osteoclasts leading to increased osteoclastogenesis and metastatic osteolytic lesions (4). In another model, formation of osteolytic lesions has been attributed to direct activation of osteoclasts through interleukin-8 secretions by the breast cancer cells (15).

In the present investigation, we observed expression of RANK in normal breast epithelium as well as in tumor cells invading bone (Fig. 2), whereas absence of RANKL expression in the tumor cells correlated very well with the metastatic phenotype. Although this analysis only examined the phenotype of RANK and RANKL expression by immunohistochemistry, it is possible to hypothesize that the loss of RANKL expression in the tumors permitted RANK to act both as an anchor and as a stimulatory mechanism for the breast cancer cells in the bone; the loss of RANKL expression and the retention of RANK expression in the tumor would free RANK and allow it to interact with RANKL present on the osteoblasts and stromal cells at the site of metastasis. This could act as a targeting mechanism for the tumor cells to allow them to interact and remain at the bone surface. It could also act to stimulate osteoblasts and stromal cells, which could in turn result in secondary activation of osteoclasts, leading to increased osteolysis at the site of metastasis. In support of this notion, it has been reported that the breast cancer cell line MDA-231 stimulates osteoclast formation by secreting macrophage colony-stimulating factor and by enhancing the expression of RANKL in stromal cells (12).

The observed correlation between absence of RANKL in the tumor cells and metastatic phenotype was observed in a cross-section of samples from several institutions and across a wide age range, suggesting that this may be a common mechanism for targeting breast cancer tumor cells to bone. Bone provides a fertile environment for metastases. Breakdown of the extracellular matrix by osteoclasts would release growth factors and cytokines that would stimulate the metastatic cells. Thus, there would be a selective advantage to activating osteoclastogenesis in the presence of tumor. Loss of RANKL expression could be an important clue in understanding the mechanism of osteolytic bone metastases in breast cancer and may be a useful diagnostic marker for the metastatic phenotype.

**ACKNOWLEDGMENTS**

We thank the Cooperative Human Tissue Network and Dr. Andres Klein-Szanto (Fox Chase Cancer Center) for providing tumor samples and Drs. A. Deshpande and Y-F. Huang for helpful discussions.

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**Fig. 2** Immunohistochemical analysis of RANK in normal breast and breast cancer metastases. Samples of NNB and BTM were stained by H&E as well as for expression of RANK by immunohistochemistry. A, H&E-stained NNB; B, same sample stained for RANK expression; C, H&E-stained BTM showing invasion of the bone; D, same sample stained for RANK expression.
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