Identification of Breast Cancer Cell Line-derived Paracrine Factors That Stimulate Osteoclast Activity

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ABSTRACT

Metastatic breast cancer causes destruction of significant amounts of bone, and, although bone is the most likely site of breast cancer metastasis, little is understood about interactions between tumor cells and bone-resorbing osteoclasts. We have investigated the paracrine factors produced by breast cancer cells that are involved in increasing osteoclast activity. We have determined by immunoassay that the human breast cancer cell line MDA MB 231 (231) cultured in serum-free medium secretes transforming growth factors type β (TGF-β) 1 and 2, macrophage colony-stimulating factor (M-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL) -1 and -6, tumor necrosis factor α (TNF-α), insulin-like growth factor II (IGF II), and parathyroid hormone-related peptide. To determine which of these are involved in increased bone destruction, we have fractionated serum-free 231-conditioned media and measured these fractions for effects on osteoclast resorption activity using multiple activity assays. The pattern of responses was complex. Several fractions stimulated osteoclast resorption either by increasing the number of osteoclasts binding to the bone or by elevating the resorption activity of the individual osteoclasts. Other fractions inhibited osteoclast activity. Analysis of active fractions for the factors identified in the 231-conditioned medium revealed that the presence of TNF-α and IGF-II was restricted to separate fractions that stimulated osteoclast resorption activity. The fractions that inhibited osteoclast resorption activity contained M-CSF, IL-6, TGF-β2, and GM-CSF. No TGF-β1 or IL-1 was detected in any of the active fractions. Our data support the hypothesis that breast cancer cells modulate osteoclast activity using multiple regulatory factors that increase both the number of mature osteoclasts attached to the bone and the bone resorption activity of these individual osteoclasts. Once it is understood how metastatic breast cancer elevates osteoclast-mediated bone loss, effective therapies to slow the progression and/or prevent this bone loss will become possible.

INTRODUCTION

Most women with breast cancer receive treatment that seeks to eradicate cancer cells from the breast. Twenty-four percent of those patients with an apparent permanent elimination of cancer from the breast and a lack of evidence of skeletal metastases at the time of surgery will eventually develop signs or symptoms of breast cancer metastases involving the skeleton (1). This indicates that undetectable, microscopic bone metastases were present when the breast cancer was originally diagnosed, and it underscores the importance of understanding how these microscopic breast cancer deposits in bone develop into clinically relevant tumors. Three events must occur before women with breast cancer develop the signs or symptoms of skeletal metastases: (a) cancer cells must leave the breast, travel to, and occupy the osseous intramedullary compartment; (b) cancer cells within the osseous intramedullary compartment must induce bone destruction to provide space for tumor growth; and (c) clusters of cancer cells within the osseous intramedullary compartment must grow to form solid tumors. We are investigating the hypothesis that it is locally produced, tumor-derived paracrine factors that are driving the debilitating bone loss associated with metastatic cancer.

The prevalence of bone metastasis in breast cancer patients is highlighted by the fact that, at the time of autopsy, 70% of the women who die from breast cancer show metastases to bone (reviewed in Ref. 2). Tumor cells travel to other parts of the body by altering their phenotype to exploit the blood vasculature and lymph system for transport and deposit in other tissues. Once the tumor cells arrive in bone, they can begin to grow and actively alter their environment to maximize growth. As this growth proceeds, the tumors stimulate the destruction of large amounts of bone at the site of the tumor. This focal loss of bone weakens the skeletal structure and usually results in considerable pain, decreased mobility, hypercalcemia, and significant levels of skeletal fracture. Once tumor cells are deposited and begin to grow in the bone, curative therapy is problematical. For most of these patients, the goals of treatment aim to alleviate discomfort and prevent pathological fractures. Current treatments enable control of tumors in the breast, and patient deaths are more likely caused by metastatic cancer. Thus, therapies that limit tumor-driven bone destruction could greatly slow the progression of complications and suffering. Because a significant problem both in terms of patient suffering and in terms of promoting tumor progression is the result of tumor-driven osteolysis, tumor stimulation of osteoclastic bone resorption is an important target in studies seeking for ways to slow tumor progression.

Multiple growth factors and cytokines have been reported to influence osteoclast differentiation (reviewed in Refs. 3, 4). These include M-CSF, GM-CSF, IL-1 and -6, TGF-β, IGFs, TNF-α, and PTHrP. Much less is known concerning the influences of these factors on the resorption activity of mature osteoclasts. Osteoclasts have been reported to express receptors for M-CSF, TGF-β, IGFs, IL-1, IL-6, and PTH/PTHrP (5–10). Thus, these factors could potentially impact the activity of the differentiated cells directly in addition to influencing differentiation. We have, therefore, sought to determine whether breast cancer cells stimulate osteoclast resorption directly and which growth factors secreted by these cells are candidate factors responsible for this stimulation.

MATERIALS AND METHODS

Collection of Breast Cancer Cell Line-conditioned Media

MDA MD 231 cells were obtained from American Type Culture Collection (Rockland, MD) and subcultured in phenol red-free α Minimal Essential Medium (αMEM) obtained from Life Technologies, Inc., Gaithersburg, MD, supplemented with 10% fetal bovine serum at 37°C, 5% CO₂ until confluent. Cell monolayers were washed with sterile PBS (pH 7.4) and placed in αMEM

Received 5/24/99; accepted 9/22/99.

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The abbreviations used are: M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; TGF-β, transforming growth factor β; IGF, insulin-like growth factor; TNF-α, tumor necrosis factor α; PTH, parathyroid hormone; PTHrP, PTH-related peptide; TRAP, tartrate-resistant acid phosphatase.
supplemented with 0.25% (wt/v) BSA obtained from Sigma Chemical Co. (St. Louis, MO) for 3 days. At the time of collection, cellular debris was removed by centrifugation, and aliquots were frozen at $-70^\circ$C until analyzed.

**Osteoclast Isolation and Culture**

Osteoclasts were isolated from White Leghorn hatchlings that were maintained on a low-calcium diet for a period of 5 weeks (11). All of the animals were treated as humanely as possible and treatment followed the NIH and institutional guidelines for care and use of experimental animals. An osteoclast-directed monoclonal antibody, 121F (a gift from Dr. Philip Osdoby, Washington University, St. Louis, MO), coupled to immunomagnetic beads obtained from Dynal, Inc., was used to obtain cell populations that consisted of at least 90% pure osteoclasts and 10% or less unidentified mononuclear cells (12). The purified osteoclasts exhibited all of the phenotypic attributes of osteoclasts including multinucleation, attachment, and ruffled border formation when cultured with bone particles, and the ability to attach and form resorption pits when cultured on slices of cortical bone. Osteoclasts were cultured in phenol red-free αMEM supplemented with 0.25% (wt/v) BSA as described for individual experiments (see figure legends and below).

**Resorption Analyses**

**Quantitative Pit Formation Assay.** Isolated osteoclasts were plated on 1-mm² slices of bovine cortical bone. Bone slices were prepared as described previously (13). Samples were treated with vehicle or the indicated test substance as detailed in the figure legend. After 24 h of culture, the slices were placed in 1% (v/v) paraformaldehyde in PBS. The number of osteoclasts per mm² slice was determined for each slice as follows: the fixed slices were rinsed with water and stained for TRAP activity using a Sigma histochemical kit. Osteoclasts were identified as stained multinucleated cells. The number of pits per osteoclast was determined after removal of the cells. The pits, resulting from osteoclast activity, were stained with toluidine blue, counted by reflected light microscopy, and expressed as the number of pits per osteoclast as described previously (13, 14).

**Quantitative Lysis of Collagen by ELISA.** Osteoclasts were cultured on bone slices with either vehicle or the test substance as detailed in the figure legends. The conditioned media were harvested, and the amount of antigenic collagen fragments released was determined as described previously (15).

**Lyosomal Enzyme Assays**

Cell pellet extracts and conditioned media were assayed. To standardize for relative cell number, the protein content of the solubilized cell pellet was determined using the Bio-Rad protein detection system. TRAP activity was measured using an assay based on the work of Hofstee (16). The initial rate of hydrolysis of $\alpha$-carboxy phenyl phosphate was determined by following the

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Fig. 1. Osteoclast responses to selected breast cancer cell line-conditioned media. Isolated osteoclasts were cultured with the indicated concentration of the following tumor cell line-conditioned media: MDA MB 231 (231), MDA MB 435 (435), MCF-7, and T47D, or control (CONT). Analysis was done after 24 h of culture. The number of pits formed per osteoclast per 1-mm² bone slice was determined as described in the “Materials and Methods” section. The experiment was done in triplicate, and the results are presented as the mean ± SE; *, $P < 0.05$.

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Fig. 2. Effects of human breast cancer cell line-conditioned media on osteoclast resorption activity. Isolated osteoclasts were cultured with the indicated concentration of MDA MB 231 tumor cell line-conditioned media. Analysis was done after 24 h of culture. A, the amount of collagen peptide released into the media. B, the number of pits formed per osteoclast per 1-mm² bone slice. C, the number of osteoclasts per 1-mm² bone slice. Each experiment was done in triplicate, and the results are presented as the mean ± SE; *, $P < 0.05$.

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increase in absorbency at 300 nm resulting from the liberation of salicylic acid. One unit is defined as the amount that hydrolyses 1 μmol of \( o \)-carboxy phenyl phosphate per min at 24°C (pH 5.0). The assay was performed in the presence of 1 mM tartrate. cathepsin B levels were measured by Na-CBZ-lysine \( p \)-nitrophenyl ester hydrolysis as measured by 520 nm absorbance as outlined by Barrett and Kirschke (17).

### Preparation of Growth Factors

Recombinant human growth factors were purchased from R&D (Minneapolis, MN) and reconstituted in αMEM supplemented with 0.25% (wt/v) BSA at 1000-fold the concentration used in each experiment (see figure legends). Aliquots were stored at −70°C.

### Conditioned Media Fractionation

Conditioned media were collected as outlined above and 1 ml loaded onto a Superdex 75 molecular sieve column (Pharmacia, Piscataway, N.J.), which has a functional separation range of \( M_r \) 5,000–75,000 after pre-equilibration with αMEM using 3 bed volumes (150 ml) at 1 ml/min. Gel filtration separation of the sample is carried out with a flow rate of 0.5 ml/min with a back-pressure of 0.7 Mpa. Thirty 1-ml fractions were collected on ice and frozen immediately at −70°C until assayed for effects on osteoclast activity or growth factor quantitation.

### Quantitation of Growth Factors and Cytokines

IL-1, IL-6, M-CSF, GM-CSF, TGF-β1, TGF-β2, and TNF-α were quantitated using R&D Quantikine kits according to the instructions. IGF-II and PTHrP levels were analyzed by the method of de Leon and Asmerom (18).

### Statistical Analysis

Unless otherwise indicated in the figure legends, the results represent the mean ± SE of three separate experiments. The effect of treatment was compared with control values by one-way ANOVA; significant treatment effects were further evaluated by the Fisher’s least significant difference method of multiple comparisons in a one-way ANOVA. Tests were carried out using Apple software, obtained from Statview II (Abacus Concepts, Inc., Cupertino, CA).

### RESULTS

**Breast Cancer Cell Line-conditioned Media Studies.** Initially, we surveyed conditioned media from several well-characterized breast cancer cell lines for their effects on osteoclast resorption activity. As demonstrated in Fig. 1, conditioned media from each of these cell lines stimulated bone resorption, although the stimulatory level varied with the cell line. For subsequent studies, we have focused our studies on the cell line MDA MB 231 because this cell line has proven to cause osteolytic lesion in an \( in vivo \) animal model (19). To estimate the total volume of bone resorbed when osteoclasts are cultured in the presence of MDA MB 231 cell-conditioned media, we have used a newly developed assay that quantitates the amount of collagen peptide released. Osteoclasts were cultured on bone slices and treated with a series of dilutions of MDA MB 231 cell-conditioned media (Fig. 2A). This analysis revealed that there was a dose-dependent effect of the conditioned media on osteoclast activity. Interestingly, the response was biphasic, with a maximal effect at a 0.1% dilution of the conditioned media and an inhibitor effect at the

<table>
<thead>
<tr>
<th>Factor</th>
<th>Concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>31.7 ± 3.6</td>
</tr>
<tr>
<td>IL-6</td>
<td>45.2 ± 7.5</td>
</tr>
<tr>
<td>M-CSF</td>
<td>14,900 ± 1,245</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>152.2 ± 54</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>1,237.8 ± 97.1</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>6,895.4 ± 2,437</td>
</tr>
<tr>
<td>TNF-α</td>
<td>31.1 ± 5.3</td>
</tr>
<tr>
<td>IGF-II</td>
<td>1,000 ± 50</td>
</tr>
<tr>
<td>PTHrP</td>
<td>35,000 ± 7,000</td>
</tr>
</tbody>
</table>

![Table 1 Growth factor/cytokine analysis of conditioned media](https://cancerres.aacrjournals.org/)

**Fig. 3.** Size fractionation of MDA MB 231-conditioned media. Conditioned media (0.5 ml) was fractionated and added to aliquots of freshly isolated osteoclasts on slices of bone after filter sterilization. Analysis was done after 24 h of culture. Results are presented for A, the amount of collagen peptide released into the media; B, the number of pits formed per osteoclast per 1-mm² bone slice; C, the number of osteoclasts per 1-mm² bone slice. Each experiment was done in triplicate, and the results are presented as the mean ± SE; *, \( P < 0.001 \).
highest dose. Using the pit formation assay, we observed a biphasic effect of the conditioned media, but the peak stimulation in the number of pits per osteoclast was at a dilution of 1% conditioned media (Fig. 2B). Again, the highest dilution was inhibitory. A similar pattern emerged when the number of osteoclasts per bone slice was assessed with the peak concentration at 0.1% conditioned media (Fig. 2C). Having ascertained that MDA MB 231 cells produced a substance or substances that stimulated osteoclast activity, the conditioned media were assayed for the presence of a number of cytokines and growth factors (Table 1). Significant levels of IL-1, IL-6, M-CSF, GM-CSF, TGF-β1, TGF-β2, TNF-α, IGF-II, and PTHrP were measured in the conditioned media.

Identification of Candidate Stimulatory Factors. The above list contained many factors that could be working either alone or in combinations to stimulate osteoclast activity. To further define the list of candidate osteoclast stimulatory factors, MDA MB 231-conditioned media were passed over a molecular sieve column under nondenaturing conditions. The resultant fractions were sterile filtered and assessed for effects on osteoclast activity. The pattern of effects on the total amount of collagen peptide released into the media suggested that there were regions that stimulated resorption and, interestingly, regions that repressed osteoclastic activity (Fig. 3A). When the number of pits per osteoclast was examined, there were several fractions that stimulated bone resorption but no regions that appeared to inhibit the number of pits formed per cell (Fig. 3B). When the number of osteoclasts per slice was examined, fractions that had elevated collagen-releasing effects but no effect on the number of pits per slice contained more cells per slice (Fig. 3C). Similar to the pattern observed when the amount of collagen peptide released was determined, there were fractions that had fewer osteoclasts per slide than control cultures. These assays have indicated that there were stimulatory and inhibitory conditioned media fractions. Both stimulatory and inhibitory fractions were assessed for the presence of the same growth factors that were identified in Table 1. As detailed in Table 2, TNF-α and IGF-II were present in fractions that stimulated resorption. GM-CSF, M-CSF, IL-6, TGF-β2, and PTHrP were present in the fractions that inhibited osteoclast activity. No IL-1 or TGF-β1 was measured in any of the active fractions. All of the active fractions were examined for cytokine and growth factor levels, and several of the active fractions contained no detectable levels of any of the factors examined.

Effects of Growth Factors on Osteoclast Activity. We examined the effects of the above identified factors on osteoclast resorption activity (Fig. 4). IGF-II, PTHrP, and TNF-α each stimulated resorption activity by all of the parameters measured here. TGF-β2 stimulated the number of pits per osteoclast and the number of osteoclasts per slice, but the total volume of collagen peptide released was not significantly altered with treatment. In contrast, treatment with GM-

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**Table 2 Growth factor and cytokine analysis of fractions**

All active fractions of the MDA MB 231-conditioned media were analyzed in triplicate for all of the growth factors listed in Table 1. The fractions listed below were positive for the indicated growth factors and the concentrations (mean ± SE of three replicates) are given in parentheses. This experiment was repeated a total of three times, and these results are representative of the levels detected.

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Factor(s) (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>TNF-α (14 ± 1.1)</td>
</tr>
<tr>
<td>10</td>
<td>IGF-II (12 ± 2.3)</td>
</tr>
<tr>
<td>13</td>
<td>M-CSF (362 ± 68); IL-6 (34 ± 7); PTHrP (7,000); TGF-β2 (18,260 ± 75)</td>
</tr>
<tr>
<td>14</td>
<td>GM-CSF (128 ± 18); M-CSF (792 ± 530); IL-6 (112 ± 14); TGF-β2 (41,590 ± 1,276)</td>
</tr>
<tr>
<td>15</td>
<td>GM-CSF (297 ± 13); M-CSF (3,168 ± 184); IL-6 (924 ± 17); TGF-β2 (6,400 ± 527); PTHrP (5,000)</td>
</tr>
<tr>
<td>16</td>
<td>GM-CSF (86 ± 17); IL-6 (496 ± 21)</td>
</tr>
</tbody>
</table>

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Fig. 4. Analysis of selected factor effects on bone resorption activity. Isolated osteoclasts were cultured with either vehicle (media with 0.25% BSA) or the indicated factors at the following concentrations for 24 h: GM-CSF, 0.15 ng/ml; IGF-II, 10 pg/ml; PTHrP, 5 ng/ml; TNF-α, 15 pg/ml; TGF-β2, 50 ng/ml; M-CSF, 3 ng/ml; IL-6, 500 ng/ml. The concentration was selected with reference to Table 2. Results are presented for A, the amount of collagen peptide released into the media; B, the number of pits formed per osteoclast; and C, the number of osteoclasts per 1-mm² bone slice. The experiment was done a total of three times, and these are representative results. *, P < 0.05; **, P < 0.01 relative to control.
of three times, and these are representative results. Results are mean media. Assays were performed on seven slices/treatment. The experiment was done a total cathepsin B activity in the conditioned media and B A The concentration was selected with reference to Table 2. Results are presented for

We were unable to detect any significant effect of M-CSF on resorption activity, we next determined whether the factors likewise

CSF significantly inhibited resorption activity as measured by determining the number of pits per osteoclast, whereas there seemed to be no significant effects on the number of osteoclasts per slice or on the total amount of collagen released. IL-6 treatment increased the number of pits per slice; yet there was no significant effect on either the amount of collagen peptide released or on the activity per osteoclast. We were unable to detect any significant effect of M-CSF on resorption activity during the 24-h treatment period by any of the criteria measured here. Having measured effects of these growth factors on resorption activity, we next determined whether the factors likewise influenced lysosomal enzyme secretion. As demonstrated in Fig. 5, IGF-II, PTHrP, TNF-α, TGF-β2, and IL-6 stimulated cathespin B and TRAP secretion when results were normalized for respective sample cell numbers. Treatment with GM-CSF or M-CSF had no effect on the secretion per osteoclast of cathepsin B or TRAP.

**Effects of Growth Factor Combinations on Osteoclast Activity.** Having assessed the effects of treatment with individual growth factors on osteoclast resorption and lysosomal enzyme activity, we explored the influences of the combinations of growth factors present in the conditioned media fractions that had either stimulatory or inhibitory effects (Table 3). The combination of IGF-II, TGF-β2, PTHrP, and TNF-α approached the stimulatory level of 0.1% conditioned media with respect to effects on bone resorption and lysosomal enzyme secretion. Interestingly, the combination of GM-CSF, M-CSF, IL-6, PTHrP, and TGF-β2 inhibited these same parameters. Combining stimulatory IGF-II and TNF-α with these inhibitory factors resulted in stimulatory activity, but the level of stimulation did not approach that of the diluted conditioned media.

**DISCUSSION**

The data presented here demonstrate that breast cancer cells secrete multiple growth factors that have the ability to stimulate osteoclast-mediated bone loss. We have shown that all of the cell lines examined secrete factors that stimulate osteoclast resorption activity. For the remaining studies, we elected to examine the MDA MB 231 cell-conditioned media because these have proven to be highly metastatic to bone using an animal model system pioneered by Nakai et al. (19). We have examined three different resorption parameters for these studies. Quantification of the total amount of bone removed was achieved by determining the amount of collagen peptide released into the media during the resorption process. This assay detects the total amount released whether it is due to increased numbers of osteoclasts, increased number of pits generated by each osteoclast, or increased pit size. Analysis of the number of pits per osteoclast indicated the activity per cell, and the calculation of the number of osteoclasts per bone slice indicated the number of cells that were present. Changes in this last parameter could be due to a number of different effects including decreased apoptosis or increased binding to bone. Resolution of the mechanisms by which the number of osteoclasts present were altered is not revealed by these studies and remains to be resolved with further experimentation. The conditioned media effects were seen at surprisingly low concentrations and were biphasic. Our studies revealed that all of the measures of osteoclast activity, including the total amount of bone removed, the activity of each osteoclast, and the number of osteoclasts bound to the bone, exhibited this biphasic response. There are several possible reasons for this; among the possibilities: depletion of important factors by using spent media or a toxic metabolic waste build up. Depletion of important factors is unlikely given the identification of inhibitory fractions after chromatography in fresh media. Any accumulated toxic metabolic products would elute as small molecules in late fractions. The inhibitory effects approached the stimulatory level of 0.1% conditioned media.

The results of this study show that breast cancer cells secrete multiple growth factors that have the ability to stimulate osteoclast-mediated bone loss. These factors include IGF-II, TGF-β2, PTHrP, and TNF-α, and their combination results in a biphasic response. The conditioned media effects were seen at surprisingly low concentrations and were biphasic. Our studies revealed that all of the measures of osteoclast activity, including the total amount of bone removed, the activity of each osteoclast, and the number of osteoclasts bound to the bone, exhibited this biphasic response. There are several possible reasons for this; among the possibilities: depletion of important factors by using spent media or a toxic metabolic waste build up. Depletion of important factors is unlikely given the identification of inhibitory fractions after chromatography in fresh media. Any accumulated toxic metabolic products would elute as small molecules in late fractions. The inhibitory fractions were in midrange (estimated sizing between M, 40,000 and 100,000), indicating that this is not a likely explanation either.

Not surprisingly, there were many growth factors and cytokines present in the conditioned media. To better define the growth factors in the conditioned media that were active in stimulating osteoclast activity, the media was fractionated using an approach that was not disruptive to native protein conformations and interactions. Analysis of these fractions with the three different resorption activity measurements has revealed an interesting pattern of responses that varies according to which resorption criteria was examined. As outlined in Table 4, fractions 3, 4, and 5 increased the total amount of bone removed and the activity of each osteoclast. In these fractions, the...
Isolated osteoclasts were treated with vehicle, conditioned media, the growth factors that individually stimulated osteoclast activity in the concentrations as indicated in Fig. 4, and/or the growth factors that are present in the inhibitory fractions of conditioned media as detailed in Table 2 (GM-CSF + IL-6 + PTHrP + TGF-β). The cultures were analyzed as detailed in the "Materials and Methods" section. The experiment was repeated three times, and the data are from one of these experiments and represent typical results. Data are the mean ± the SE of the replicates from one experiment.

We examined all of these fractions to determine the presence of IL-1, IL-6, M-CSF, GM-CSF, TGF-β1, TGF-β2, TNF-α, IGF-II, and PTHrP. In the stimulatory fractions, we detected TNF-α in fraction 6 and IGF-II in fraction 10. None of the other fractions, whether stimulatory or inhibitory contained these factors. As indicated in Table 2, some of the inhibitory fractions contained GM-CSF, M-CSF, IL-6, TGF-β2, and PTHrP. Other inhibitory fractions contained no detectable levels of the factors examined. This is intriguing, and we are presently pursuing the content of these fractions with more extensive studies. None of the fractions contained detectable levels of TGF-β1 or IL-1, which suggests the possibility that these factors were diluted by the fractionation sufficiently to be below the detection limits of the assays (TGF-β1: <7 pg/ml; IL-1: 0.5 pg/ml) or they were present in untested, therefore inactive, fractions.

Because several of the factors present in the inhibitory fractions have been shown to stimulate osteoclastic resorption, we examined whether the factors identified in the stimulatory fractions were capable of stimulating osteoclasts in our system. Our studies revealed that human IGF-II and TNF-α both stimulated the activity of the avian osteoclasts. Because these were identified in two of the stimulatory fractions, it seems likely that these factors are breast cancer-derived factors that are involved in stimulating osteoclast-mediated bone resorption. Hou et al. (7) have demonstrated that purified rabbit osteoclasts have IGF-I receptors, bind IGF-I with high affinity, and respond to IGF-I treatment with decreased apoptosis. In contrast with these findings, others have shown that IGFS either have no effect or stimulate only if osteoblasts are present (20, 21). TNF-α stimulates osteoclast differentiation, but the effects on mature cells have not been extensively studied (3, 4). TNF-α receptor 1 knockoutseem to have normal bone, which suggests that TNF-α has little role in normal bone development but does not preclude a role in pathological bone loss (22). IGF-II, TGF-β, PTHrP, and TNF-α each individually stimulated osteoclast activity. The addition of these factors together (in concentrations similar to that found in diluted 231-conditioned media) stimulated osteoclast activity to a level approaching that of the diluted conditioned media.

Interestingly, the effects of the factors identified in the inhibitory fractions were more complex. Individually, several of the factors stimulated the activity of the isolated osteoclasts, whereas neither

### Table 3 Effects of growth factor combinations on osteoclast activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Collagen nm</th>
<th>Pits/OC</th>
<th>No. of OC</th>
<th>CATH B</th>
<th>TRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1.7 ± 0.2</td>
<td>0.46 ± 0.09</td>
<td>43 ± 7</td>
<td>34 ± 9</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td>0.1× conditioned media</td>
<td>9.2 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.87 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38 ± 12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>290 ± 28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.2 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IGF-II + TGF-β + PTHrP + TNF-α</td>
<td>9.7 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.91 ± 0.14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>103 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>258 ± 27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.6 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GM-CSF + IL-6 + PTHrP + TGF-β</td>
<td>0.4 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.27 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>39 ± 9</td>
<td>12 ± 8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.8 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TNF-α + IGF-II + GM-CSF + IL-6 + PTHrP + TGF-β</td>
<td>5.1 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.36 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>55 ± 7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>87 ± 16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.3 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> OC, osteoclast; CATH B, cathepsin B.
<sup>b</sup> P < 0.001 comparing vehicle to treatment.
<sup>c</sup> P < 0.01 comparing vehicle to treatment.

### Table 4 Comparative analysis of conditioned media fractions

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Total volume of bone removed</th>
<th>ACT&lt;sup&gt;a&lt;/sup&gt;/cell</th>
<th>OC no.</th>
<th>Regulatory factor causing response</th>
</tr>
</thead>
<tbody>
<tr>
<td>3–5</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>OC ACT/cell</td>
</tr>
<tr>
<td>6, 7</td>
<td>↑</td>
<td>↔</td>
<td>↑</td>
<td>No. of OCs bound</td>
</tr>
<tr>
<td>9, 10</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>Counter balance OC ACT/cell and no. of OCs bound</td>
</tr>
<tr>
<td>11–20</td>
<td>↑</td>
<td>↔</td>
<td>↓</td>
<td>↑ No. of OC bound ? Pit volume</td>
</tr>
<tr>
<td>23, 24</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>↑ Pit volume</td>
</tr>
</tbody>
</table>

<sup>a</sup> ACT, activity; OC, osteoclast.
GM-CSF nor M-CSF stimulated activity. Indeed, GM-CSF decreased the activity of individual osteoclasts. There is little data available on GM-CSF effects on mature osteoclast activity, but M-CSF has been implicated in suppressing apoptosis (23). IL-6 significantly increased the number of osteoclasts per slice, whereas it had no effect on the activity per cell or the amount of collagen peptide released. Because the major effects of IL-6 seem to be on osteoclast differentiation and we are examining highly purified mature osteoclasts, a lack of effect of IL-6 on resorption activity is not surprising (24). In seeming contradiction to these results, it has been shown that mature osteoclasts have IL-6 receptors and that IL-6 reverses calcium-induced decreases in bone resorption (25). Direct effects of PTHrP on osteoclast activity have not been reported, but PTH, which uses the same receptor, seems to directly alter F actin distribution and cytosolic pH (8, 26). TGF-β influences on osteoclast activity are somewhat mixed, with demonstrations of stimulation and inhibition of resorption and also a stimulation of apoptosis (27, 28). Thus, studies of direct growth factor effects on resorption activity are in their infancy, inasmuch as highly purified authentic cells are not routinely used for these studies, and receptor identification studies are just now being undertaken. In our studies, the combination of the factors found in the fractions that decreased bone resorption (GM-CSF, M-CSF, IL-6, PTHrP, and TGF-β) were inhibitory, supporting the idea that the inhibitory factors present were able to overcome the stimulatory effects of some of the components of the mixture. When IGF-II and TNF-α were added to this inhibitory mixture, there was stimulation of resorption activity, but the level did not approach the stimulatory effects of the conditioned media. This leads us to conjecture that there are other stimulatory factors being secreted by the tumor cells that we have not identified. This possibility is strengthened by the presence of stimulatory fractions in which we were unable to detect the factors we are studying.

Taken together, the data presented here demonstrate that metastatic breast cancer tumors are likely to produce multiple factors that have diverse effects on osteoclast bone resorption activity. The effect of some of the secreted factors in suppressing bone resorption was unexpected, but our data clearly show that the overall effect of the combination of inhibitory and stimulatory factors was stimulatory. This is based on both the conditioned media studies and the effects of combined stimulatory and inhibitory purified growth factor studies. These data support the possibility that IGF-II and TNF-α are likely to be key factors secreted by metastatic breast cancer tumors responsible for stimulating bone resorption activity.
Identification of Breast Cancer Cell Line-derived Paracrine Factors That Stimulate Osteoclast Activity

Larry Pederson, Bent Winding, Niels T. Foged, et al.


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