AD_____

Award Number: DAMD17-97-1-7157

TITLE: Breast Cancer Stimulation of Osteolysis

PRINCIPAL INVESTIGATOR: Merry Jo Oursler, Ph.D.

CONTRACTING ORGANIZATION: University of Minnesota
Duluth, Minnesota 55812-2496

REPORT DATE: September 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-97-1-7157
Organization: University of Minnesota

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

Earl Grant Jr. _____ _____

21 Feb. 01 _____ _____

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2000	Final (1 Sep 97 - 31 Aug 00)

4. TITLE AND SUBTITLE
Breast Cancer Stimulation of Osteolysis

5. FUNDING NUMBERS
DAMD17-97-1-7157

6. AUTHOR(S)
Merry Jo Oursler, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Minnesota
Duluth, Minnesota 55812-2496

E-MAIL:
moursler@d.umn.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Sep 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

In the three years of funding, we have completed analysis of two tumors and have found mRNA and protein for GM-CSF, IGF-I and II, PTHrP, and TNF-α. We have also studied a mouse model for metastatic tumor development and determined that there is variable timing of the appearance of tumor-derived factors during tumor development. We have examined these factors for their effects on apoptosis and found that TNF-α, but not GM-CSF, IGF-I or II, or PTHrP stimulate apoptosis of purified mouse osteoclast-like cells. We have also determined that TNF-α, but not GM-CSF, IGF-I or II, or PTHrP act as survival agents for osteoclast-like cells. When osteoclast-like cells differentiate in the presence of TNF-α, withdrawal of TNF-α once the mature cells are purified induces apoptosis while continued treatment with TNF-α represses apoptosis. We have also examined the activity of cells that are differentiated in the presence of PTHrP and TNF-α for resorption activity and lysosomal enzyme secretion. Both treatments, either alone or in combination, result in more active osteoclast-like cells. All treatments resulted in increased resorption and secretion of cathepsin B, but only the cells that differentiated in the presence of TNF-α had elevated secretion of TRAP. Taken together

14. SUBJECT TERMS
Breast Cancer Osteolysis osteoclast apoptosis survival bone resorption

15. NUMBER OF PAGES
45

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

The purpose of this research is to examine granulocyte macrophage colony stimulating factor (GM-CSF), insulin-like growth factor II (IGF-II), tumor necrosis factor alpha (TNF-α), and parathyroid hormone related peptide (PTHrP) are present at the sites of breast cancer metastases in bone and determine the mechanisms by which breast cancer cells stimulate the activity of isolated osteoclasts *in vitro* by studying the effects of GM-CSF, IGF-II, TNF-α, and PTHrP on resorption activity, apoptosis, integrin expression, and lysosomal enzyme secretion. As detailed in the accompanying report, we have made excellent progress in these studies. We have determined that these factors are present in tumors and that they have variable effects on osteoclast resorption, lysosomal enzyme secretion, and apoptosis. Much of our most recent work has focused on the influences of TNF-α on osteoclast survival. We have important new data that osteoclast that differentiate in the presence of TNF-α become dependant on TNF-α for survival while osteoclast that differentiate without exposure to TNF-α rapidly apoptose when treated with TNF-α once mature and purified. These are important findings as they may lead the way for more effective therapies to block tumor-driven osteolysis in tumors that metastasize to bone.

BODY

Overall, as detailed below, we have made excellent progress on this study. Many of the tasks are either completed and published (reprint appended) or are in the process of being submitted for publication. This report is detailed with specific reference to the Statement of Work.

Specific Aim 1: Determining if GM-CSF, IGF II, TNF-α, and PTHrP are present at the sites of tumor metastasis and likely to be involved in stimulating osteoclast activity.

Task 1: 1-12 months: PCR analysis of tumors.

Human patient sample work was completed in year 1 and included in the previous progress report. The results of this aspect of the project are currently being incorporated into a manuscript. We have expanded these studies by using an animal model for breast cancer metastasis.

METHODS

Materials

MDA MB 231 human breast cancer cells were obtained from ATCC and ST2 mouse stromal cells were obtained from Riken (Ibaraki, Japan). Cells were grown to confluence in alpha-modified minimum essential medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotic/antimycotic solution (Sigma, St. Louis, MO) in 5% CO_2 at 37°C. Prior to harvesting RNA, adherent cells were washed gently with phosphate-buffered saline, pH 7.4. Osteoclast precursors were harvested by flushing the marrow from femurs of male Balb/C mice (Taconic, Germantown, NY) as described previously (1). Marrow cells were centrifuged, resuspended in media containing 12% dimethylsulfoxide, and frozen in liquid nitrogen until use. Spleens were harvested from the mice and frozen at -70°C until use. 1, 25-dihydroxyvitamin D_3 was purchased from Biomol (Plymouth Meeting, PA) and resuspended in ethanol at a concentration of 10^{-4} M. Dexamethasone was purchased from Sigma and resuspended in ethanol at a concentration of 10^{-4} M.

Cardiac injections

Cardiac injections were performed as modified (2) at the OsteoPro A/S Center for Clinical and Basic Research. Briefly, MDA MB 231 cells were injected into the left cardiac ventricles of female nude mice. Animals were treated as humanely as possible and maintained under government guidelines for care and use of experimental animals. Tumors were allowed to develop for 1, 2, 3, or 4 weeks before bones were harvested for analysis. Prior to harvesting bones, the number and size of osteolytic lesions was determined by radiography. Mouse femora and tibiae were harvested and stored at -70°C until analysis. A total of 45 animals were evaluated in this study. This represented 2 animals at 0 weeks, 8 animals at 1 week, 7 animals at

2 weeks, 7 animals at 3 weeks, 10 animals at 4 weeks after tumor cell injection, and 11 animals that served as controls receiving vehicle injections.

Generation of mouse osteoclast-like cells

Mouse osteoclast-like cells (mOCLs) were generated from mouse marrow precursors using the method described previously (1). ST2 mouse stromal cells served as support cells and were grown to confluence in alpha-modified minimum essential medium (Sigma) supplemented with 10% fetal bovine serum (Hyclone) at 37°C, 5% CO_2. ST2 cells were then plated into 48 well plates at a density of 8 x 10^4 cells per well and allowed to attach for 8-18 hours. Osteoclast precursors were thawed into phenol red-free minimum essential medium supplemented with 10% FBS, and 50 ng/ml ascorbic acid. 1, 25-dihydroxyvitamin D_3 and dexamethasone were added to achieve a final concentration of 10^{-7} M each, and precursors were plated at a density of 5 x 10^4 cells per well onto the attached ST2 cells. Media were changed every third day and fresh 1, 25-dihydroxyvitamin D_3, dexamethasone, and ascorbic acid were added. Presence of mOCLs was verified by staining for tartrate-resistant acid phosphatase (Sigma) and observation of multinucleated cells.

Preparation of RNA

Since tumor burden in each bone varied, RNA was isolated from both femora and tibiae from each animal and combined before analysis. Total RNA was isolated from mouse bones by first crushing bones in liquid nitrogen and then extracting RNA using the acid guanidinium thiocyanate-phenol-chloroform method (3). RNA was further purified by lithium chloride precipitation and ethanol precipitation. RNA was then subjected to DNase digestion for 1 hour to degrade contaminating genomic DNA. DNase and residual nucleotides were removed by phenol-chloroform extraction followed by ethanol precipitation. The purified RNA was quantitated by UV spectrophotometry before analysis by reverse transcription polymerase chain reaction (RT-PCR), Northern blotting, or ribonuclease protection assay (RPA).

RT-PCR (Reverse Transcription-Polymerase Chain Reaction)

Prior to PCR, cDNA was generated from mRNA by reverse transcription. 5 μg of total RNA was denatured by heating to 90°C for 90 seconds. Then RNA was incubated with 10 units M-MLV reverse transcriptase, 50 μg/ml oligo dT primer (Gibco BRL), 40 μM dNTP mix, and RNase inhibitor (Promega, Madison, WI) in the buffer provided with the enzyme for 90 minutes at 42°C. Following reverse transcription, cDNA was purified by phenol-chloroform extraction followed by ammonium acetate-ethanol precipitation to degrade RNA. PCR primers were designed to span an intron to enable us to distinguish between genomic DNA amplification and cDNA amplification based on size differences in the amplicon. PCR primers were chosen using gene sequences available on GenBank (see Table 1). Due to highly conserved gene sequences between mice and humans, species-specific primers could not be designed for all growth factor transcripts. PCR mixes contained 40 μM dNTP mix, 1.5 mM $MgCl_2$, 50 μg/ml each primer, and 1.25 U AmpliTaq Gold Taq polymerase (Perkin-Elmer). PCR was carried out under the following conditions: denature for 1 minute at 94°C, anneal primers for 1 minute at 55°C, and extend for 3 minutes at 72°C for 40 cycles. PCR products were then separated by gel electrophoresis on a 2% NuSieve 3:1 agarose gel (FMC BioProducts) and stained with ethidium bromide. Bands were visualized under UV light and photographed.

Northern blotting

10 μg of total RNA was denatured by incubating in a solution containing 0.8 M glyoxal, 40% dimethylsulfoxide, 0.008 M NaH_2PO_4 for 1 hour at 55°C. Denatured RNA was separated by electrophoresis on a 1% SeaKem LE agarose gel (FMC Bioproducts, Rockland, ME). RNA was

then transferred by capillary action to a Hybond-N nylon filter (Amersham, Piscataway, NJ) and crosslinked by baking at 85°C for 4 hours under vacuum. Human TGF-β_1 cDNA was generously provided by Rick Derynck (Genentech, South San Francisco, CA). Probe was [α-^{32}P]dCTP-labeled using the Random Primer Extension Labeling System (NEN, Boston, MA). Blots were prehybridized for 12 hours at 42°C in a solution containing 1.5% formamide, 0.03% Denhardts (1% each polyvinylproladine, Ficoll, BSA), 0.003 mg/ml poly A, 0.03% SDS, and 0.05 mg/ml sheared salmon sperm DNA in SSC (3M NaCl, 0.3 M sodium citrate). Blots were hybridized in the same solution with 2 x 10^7 cpm labeled probe at 42°C for 4 hours. Blots were washed twice with warm 0.5X SSC, 0.1% SDS at 42°C for 15 minutes. Blots were then allowed to dry and RNA-cDNA hybrids were visualized by autoradiography.

RPA (Ribonuclease Protection Assay)

RPA probe templates were purchased from BD PharMingen. [α-^{32}P]UTP-labeled RNA probes were generated using *in vitro* transcription according to manufacturer's protocol (BD PharMingen). RNA probes were hybridized with mouse bone RNA by heating to 90°C and cooling overnight to 56°C by ramping at a rate of approximately 8°C per hour in a thermal cycler. Hybrids were then incubated at 56°C for 8-12 hours. RNase treatment was carried out according to manufacturer's protocol (BD PharMingen) and samples were purified by phenol-chloroform extraction and ethanol precipitation. Fragments were separated by denaturing polyacrylamide gel electrophoresis (5 % acrylamide/bis final 19:1, 48% urea). The gel was dried for 1 hour at 90°C and bands were visualized by autoradiography. The identity of the various protected fragments was determined by comparison with the undigested probe and the provided positive control RNA.

Quantitation

Quantitative RPA analysis was carried out by densitometry using NIH Image 1.61 to measure the density of protected fragment bands. Differences in RNA loading were normalized by standardizing according to GAPDH and L32 housekeeping gene bands.

Statistical Analysis

Datapoints were compared using a one-tailed Student's *t*-test. Differences were considered to be statistically significant at a 0.05 level of significance.

RESULTS

Development of osteolytic lesions *in vivo*

Osteolytic lesions became evident in mice 2 weeks after tumor cell injection. Lesions increased in size and frequency by weeks 3 and 4. Numerous osteolytic lesions were present in all but one mouse 4 weeks after tumor cell injection. Refer to Table I for a summary of X-ray analysis of tumor burden in mice. RT-PCR and RPA analysis were performed on separate batches of mice that were treated similarly.

RT-PCR

Specificity of RT-PCR primers

The highly sensitive RT-PCR method was used to detect expression of growth factors in mouse bones containing human tumor cells. The sensitivity of PCR theoretically enables one to detect gene expression at the single transcript level. We used the stringency of PCR to detect differences in human and mouse gene expression, based on mRNA sequence differences. Primers were chosen to anneal with the least homologous regions of the transcripts of interest in order to allow species-specific amplification of gene products. Due to strong homology in some of the genes we analyzed, we were not able to design species-specific RT-PCR primers for all of

the growth factors we studied. RT-PCR amplification of mouse spleen RNA and MDA MB 231 RNA with IGF-II primers revealed that the primers could amplify mouse transcripts, as well as human IGF-II transcripts. RT-PCR with GM-CSF primers revealed bands of different sizes for mouse spleen and MDA MB 231 RNA. Comparison of the mouse and human GM-CSF mRNA sequences showed that the mRNA sequence for mouse GM-CSF is 23 base pairs longer than human GM-CSF in the region flanked by the primers (4). Similarly, RT-PCR with TNF-α primers revealed bands of different sizes for mouse spleen and MDA MB 231 RNA. Comparison of mouse and human mRNA sequences showed that the human TNF-α transcript is 16 base pairs longer than the mouse transcript (5,6). RT-PCR amplification of PTHrP in mouse spleen and MDA MB 231 cells revealed that these primers recognize both mouse and human PTHrP transcripts. We designed separate primers sets to detect either mouse or human cathepsin K transcripts. Human cathepsin K primers detected cathepsin K expression in MDA MB 231 human breast cancer cells, but not mouse osteoclast-like cells. Mouse cathepsin K primers detected cathepsin K expression in mouse osteoclast-like cells, but not MDA MB 231 cells.

Expression of growth factors during osteolysis

Analysis of the pattern of expression of IGF-II, GM-CSF, TNF-α, and PTHrP revealed that these growth factors are differentially expressed throughout the development of osteolytic lesions (Figure 1). Incidence of expression of mouse GM-CSF declined, while expression of human GM-CSF increased with increasing tumor size. Interestingly, most of the samples that did not express mouse GM-CSF expressed human GM-CSF, suggesting that a factor expressed by the tumor may repress marrow cell expression of GM-CSF and allow expression of GM-CSF by the tumor. IGF-II showed a high incidence of expression, although it was not expressed in all samples. Expression of PTHrP also seemed to correlate to tumor growth, with a peak at 3 weeks and a subsequent decline after 4 weeks of tumor growth. Mouse cathepsin K was expressed in all samples, indicating that osteoclasts were present throughout the tumor development period. Increased incidence of human cathepsin K expression correlated with tumor development. The presence of actin transcripts was used to verify the integrity of the cDNA for each sample.

Northern blotting

Expression of human TGF-β_1

Human TGF-β_1 expression by the tumor correlated closely with increasing tumor size (Figure 2). 20% of mice showed expression of TGF-β_1 only 1 week after tumor cell injection. This frequency increased to 50% of mice expressing human TGF-β_1 by 4 weeks after tumor cell injection.

RPA

Specificity of RPA probes

In order to determine the specificity of RPA probes for mouse and human growth factor transcripts, we hybridized human probes with RNA generated by mouse cells known to produce the various factors. Similarly, we hybridized mouse probes with RNA from human cells known to produce the specific growth factors. RPA probes successfully detected human, but not mouse, GM-CSF, M-CSF, and LIF transcripts. Separate RPA probes successfully differentiated between mouse and human TNF-α transcripts. RPA probes were not able to effectively distinguish between mouse and human transcripts for TGF-β_1, TGF-β_2, TGF-β_3, and IL-6.

Incidence of growth factor expression during osteolysis (Figure 3)

The frequency of LIF expression increased in the later stages of osteolysis, at weeks 3 and 4. Increased expression of LIF by the tumor corresponded to an increase in tumor burden observed in the late stages of osteolysis. The frequency of IL-6 expression increased throughout

osteolysis. During weeks 2-4, all mice were expressing IL-6. It is not known whether this IL-6 originates from the tumor or from marrow cells. Human TNF-α transcripts were not detected in any mouse bone samples. However, marrow-derived TNF-α was detected in mouse bones before tumor cell injection and throughout all 4 weeks of osteolysis. Tumor-derived M-CSF transcripts were only detected during weeks 3 and 4. Transcripts for all three isoforms of TGF-β were abundant in all mouse bones analyzed, including those from mice not inoculated with tumor cells.

Quantitation of growth factor expression during osteolysis(Figure 4)
Quantitative RPA revealed that in samples that expressed a given factor, the steady state levels of mRNA for human LIF, mouse or human IL-6, human M-CSF, and human GM-CSF did not significantly change throughout osteolysis. Mouse TNF-α and all three TGF-β isoforms were expressed before tumor cell injection, and during all stages of osteolysis. Quantitative RPA showed a significant decrease in levels of mouse TNF-α transcripts after tumor cell injection, followed by a return to steady state levels during weeks 3 and 4. Quantitatively, TGF-β_1 expression did not change after tumor cell injection. TGF-β_2 expression was significantly lower during week 2, and TGF-β_3 expression was significantly lower in week 4.

DISCUSSION

These data indicate that growth factors that are capable of influencing osteoclast activity are differentially expressed during the formation of osteolytic lesions. In bone, the presence of matrix molecules impedes western blotting, so quantitative analysis of proteins is not possible. We have used species-specific RT-PCR and RPA and discovered that expression of tumor-derived GM-CSF, M-CSF, LIF, and cathepsin K is more prevalent in the latest stages of osteolysis. Expression of GM-CSF by marrow cells declines as tumor burden increases. Since GM-CSF inhibits osteoclast formation *in vitro* (7-10), repression of GM-CSF by the tumor may present a means for increasing osteoclast numbers at sites of tumor osteolysis. Since mice cannot respond to human GM-CSF secreted by the tumor (11), it is possible that repression of mouse GM-CSF expression may play a role in augmenting osteoclast formation and thereby contribute to accelerated osteolysis in this model. This species-specific effect could contribute to the observation that osteolytic lesions in these mice develop so much faster than human lesions. In humans, breast tumors in bone that secrete GM-CSF may suppress osteoclast formation to delay appearance of osteolytic lesions. Expression of M-CSF by the tumor increases as tumor burden in the mice increases. Since M-CSF is known to be an essential factor for osteoclast formation and survival (12), an increase in the number of cells expressing M-CSF could contribute to elevated osteoclast numbers *in vivo*. TNF-α was detected in mouse marrow cells throughout osteolysis. TNF-α induces osteoclast formation (13),and induces bone resorption *in vitro* (14). Since TNF-α is present during all stages of osteolysis, it may be involved in stimulation of osteoclast formation, and later in stimulation of osteoclastic bone resorption. IGF-II, and not IGF-I, is secreted by breast tumors and the MDA MB 231 cell line (15-17). IGF-I induces resorption and differentiation of osteoclasts *in vitro* (17-20). IGF-II binds and interacts with the same receptors as IGF-I (21,22), and osteoclasts express both the type I IGF receptor and the type II IGF receptor/mannose-6-phosphate receptor (17,23-25). IGF-II is present during all stages of osteolysis and may contribute to elevated osteoclast activity in the same manner as IGF-I. PTHrP expression is correlated with higher incidence of tumor-induced osteolysis *in vivo* (26). Our data suggest that PTHrP expressed by the tumor may contribute to autocrine growth of the tumor and/or osteoclast recruitment in the late stages of osteolysis. LIF is expressed by the tumor in the late stages of osteolysis. LIF induces the proliferation of osteoclast precursors (27), and osteoclast precursors possess specific receptors for LIF (28). LIF may induce proliferation of early osteoclast precursors to prevent precursor depletion due to sustained elevation of

osteoclast differentiation that may be occurring during osteolysis. Similarly, induced expression of IL-6 may contribute to osteolysis by inducing osteoclast differentiation. IL-6 stimulates osteoclast formation by inducing proliferation of early osteoclast precursors and forcing them to commit to the osteoclast line (29-31). IL-6 is not present before tumor cell injection and becomes expressed as soon as 2 weeks after tumor cell injection. This suggests that it may be an important mediator of osteolysis by stimulating osteoclast formation. TGF-β is highly expressed before and after tumor cell injection. TGF-β stimulates osteoclast formation and resorption (32,33). TGF-β may potentiate the effects of other growth factors that induce osteoclast formation and bone resorption in the area adjacent to the tumor. Expression of cathepsin K by the tumor increased as tumor burden increased. Cathepsin K is expressed by the MDA MB 231 cell line and many primary breast tumors (34). The role of cathepsin K expression by the tumor is not well understood, but it may contribute to the ability of tumor cells to invade the bone marrow compartment.

These data indicate that during breast cancer-induced osteolysis, expression of many growth factors involved in bone metabolism is altered. Perturbation of the expression patterns of growth factors may contribute to increased osteoclast-mediated bone loss that is seen in patients with metastatic breast cancer. These data are currently being prepared for inclusion in a manuscript to be submitted to Cancer Research.

Task 2: 5-16 months: Immunolocalization of factors in tumors.

We are still obtaining tumor samples for this aspect of the project. We have overcome some difficulties with developing sufficiently sensitive techniques that have low background staining. We anticipate conclusion of these studies in the final year of this project. As an alternative, we have carried out studies with our collaborators at the Center for Clinical and Basic Research in Denmark. These studies are in their early stages and work will continue on this aspect of the project with our collaborators.

Task 3: 17-36 months: examine tumors for the presence of factors.

We have discovered that explants from surgical samples secrete IGF-I and II, GM-CSF, PTHrP, and TNF-α. Table II presents the results from two of our samples. We anticipate that these studies will be included with the publication of the PCR studies (Task 1).

Specific Aim 2. Determine the mechanism by which breast cancer increase osteoclast resorption activity.

As detailed in our last progress report, we established a mammalian model system using *in vitro* generated mouse osteoclast-like cells (we had included IACUC approval notification in our first year's report). We have had concurrent studies taking place in both systems as each offered a separate strength: the avian system provided access to authentic osteoclasts and the mouse system produces mammalian osteoclast-like cells as well as having the additional benefit of allowing us to examine the influence that differentiation in the presence of the factor(s) on the phenotype of the mature cell. This has proven to be particularly important in our studies, as detailed below.

Task 4: 1-8 months: Examine the influence of GM-CSF, IGF II, TNF-α, and PTHrP on viability using the technique of fluorescein diacetate staining.

In both avian and mouse cells, we have examined GM-CSF, IGF-II, and PTHrP and have found no evidence that these influence osteoclast viability. Excitingly, we have discovered that TNF-α acts as a survival factor for mouse osteoclast-like cells that differentiate in the presence of 0.05 ng/ml TNF-α (Figure 5). We have examined the effects of withdrawal of TNF-α treatment once osteoclast-like cells differentiate and, as shown in the figure below, there are more dead cells (positive with ethidium bromide staining) in the withdrawn cultures compared to the TNF-α maintained cultures. This is being pursued as outlined in Task 5.

Task 5: 9-28 months: Examine the influence of GM-CSF, IGF II, TNF-α, and PTHrP on apoptosis with TUNEL analysis.

We are focusing on TNF-α influences on osteoclast apoptosis in the mouse system. At this time, we are pursuing studies to examine the time frame of the rapid induction of apoptosis by TNF-α as well as examining other aspects of apoptosis including membrane lipid changes (annexin V binding). We have determined that exposure to TNF-α results in apoptosis of purified osteoclast-like cells (Figure 6). This response does not require protein synthesis as it is blocked by cycloheximide treatment (Figure 7). Interestingly, mouse osteoclast-like cells from which support cells were removed exhibited rapid phosphorylation of p38 MAPK and ERK 1/2. Treatment with TNF-α suppressed ERK 1/2 phosphorylation (Figure 8). We are currently exploring the signaling pathway by which these effects are regulated.

Membrane lipid changes resulting from TNF-α treatment as an early determination of apoptosis induction (Figure 9). MOCLs from which TNF-α is withdrawn are in early stages of apoptosis compared to cells maintained in 0.05 ng/ml TNF-α. Our observation of influences of TNF-α on survival were included in an abstract that was presented at the 1999 American Society for Bone and Mineral Research meeting. We are currently using the TUNEL assay to further examine this observation.

Task 6: 1-12 months: Examine the influence of GM-CSF, IGF II, TNF-α, and PTHrP on attachment.

This aspect of the project has been concluded in avian cells and is detailed in the accompanying manuscript. Specifically, we have observed that GM-CSF has no influence on attachment whereas IGF-II, PTHrP, and TNF-α each increase osteoclast attachment. Studies in the mouse system are ongoing.

Task 7: 5-28 months: Examine effects of GM-CSF, IGF II, TNF-α, and PTHrP on on integrin expression.

On the basis of the above studies, we are currently examining the effects of GM-CSF, IGF-II, TNF-α and PTHrP on osteoclast integrin expression in the final year of funding using the mouse system. We have delayed the initiation of these studies until we completed the studies of

attachment. We are currently developing the needed sensitive staining techniques using mouse cells. We expect that these studies will be completed soon.

Task 8: 17-36 months: Examine the influence of GM-CSF, IGF II, TNF-α, and PTHrP on lysosomal enzyme secretion.

We have completed these studies using authentic avian osteoclasts and these studies are included in the appended manuscript. Specifically, we have determined the GM-CSF had no effect on lysosomal enzyme secretion. IGF-II, PTHrP, and TNF-α each stimulated secretion of cathepsin B and tartrate resistant acid phosphatase.

We are currently investigating the influence of differentiation in the presence of PTHrP, and TNF-α on mouse osteoclast-like cell bone resorption and lysosomal enzyme secretion (see Figures 10, 11, and 12). We have evidence that these factors both individually and combined increase resorption and cathepsin B secretion. Interestingly, only TNF-α increases TRAP secretion.

Table I. Tumor burden in mice. Mice were injected with MDA MB 231 human breast cancer cells and tumors were allowed to develop for up to 4 weeks. Mice were analyzed for metastatic tumors in bone and osteolytic lesions by X-ray before sacrifice.

Number of weeks post-injection	Average number of tumors/animal (# ± SEM)	Average total area of tumors (mm^2 ± SEM)
0 weeks (n = 1)	0	0
1 week (n = 3)	0	0
1 week control (n=2)	0	0
2 weeks (n = 3)	0.66 ± 0.38	0.04 ± 0.02
2 weeks control (n=1)	0	0
3 weeks (n = 3)	8.67 ± 5.00	3.38 ± 1.95
3 weeks control (n=2)	0	0
4 weeks (n = 3)	9.67 ± 5.58	3.97 ± 2.29
4 weeks control (n=2)	0	0

Table II Osteolytic breast tumors from patients were analyzed by dot blotting. Dot blots were quantitated by densitometry using NIH Image 1.60. Values were compared to a standard curve and the quantity of each growth factor present in tumor tissue and conditioned media (CM) was calculated.

	IGF-I (nM)	IGF-II (nM)	GM-CSF (nM)	TNF-α (nM)	PTHrP (nM)
tumor #1	1.93	0.84	0.10	0.60	10.05
tumor #2	2.05	2.19	0.29	0.89	14.11
tumor #2 CM	26.75	3.45	0.97	4.76	17.78

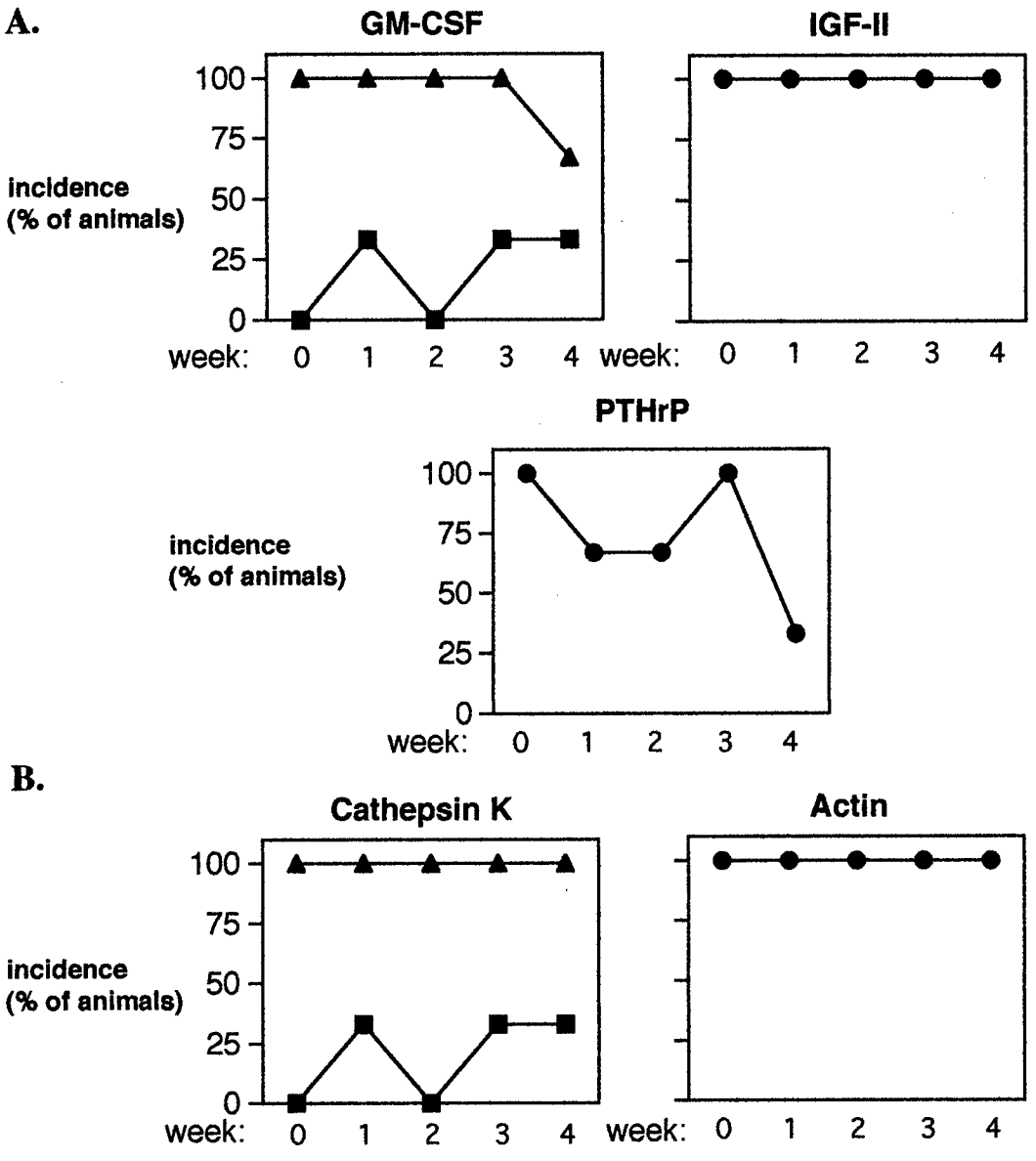


Figure 1. Growth Factor mRNA Expression. Fraction of animals showing expression of (A) GM-CSF, IGF-II, and PTHrP, or (B) cathepsin K and actin was analyzed by RT-PCR. Mouse (▲), human (■), or mouse/human (●) origin of transcripts was determined using species-specific primers. Animals were examined 0, 1, 2, 3, or 4 weeks after injection with MDA MB 231 breast cancer cells.

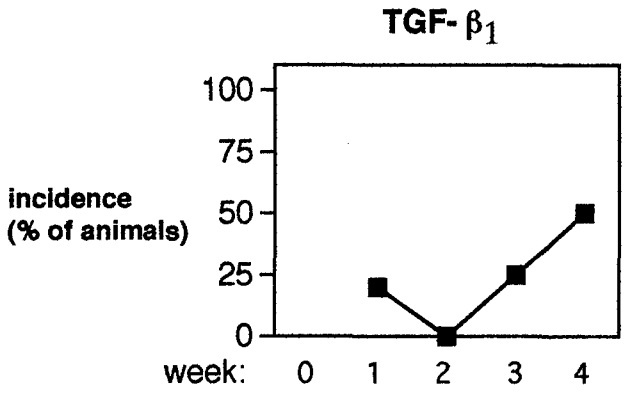


Figure 2. Fraction of animals expressing human TGFβ1 was analyzed by Northern blotting. Bones from mice inoculated with MDA MB 231 human tumor cells by cardiac injection were analyzed 1, 2, 3, and 4 weeks after tumor cell injection.

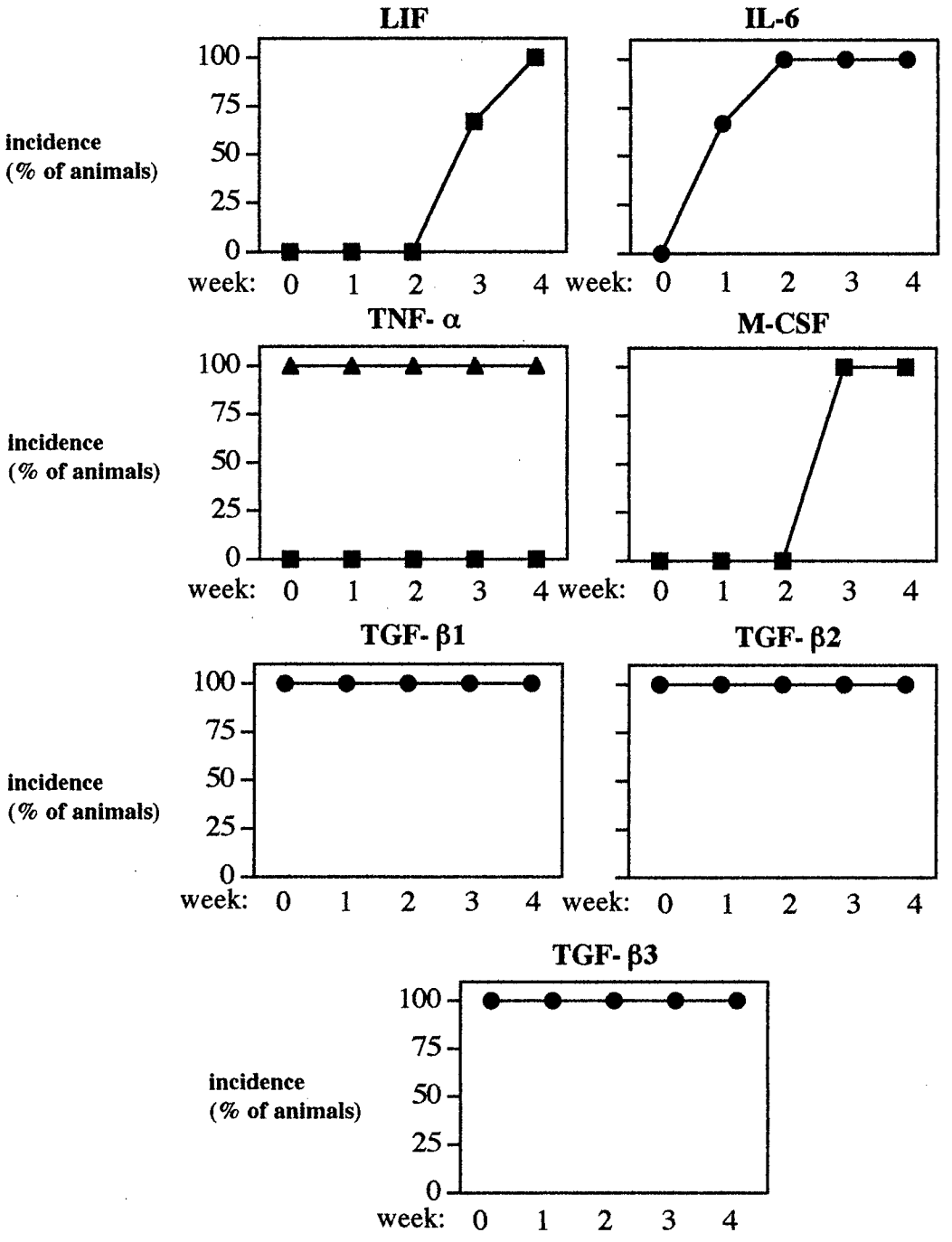


Figure 3. Incidence of growth factor expression during osteolysis. Bones from nude mice were examined 0, 1, 2, 3, or 4 weeks after cardiac injection with MDA MB 231 breast cancer cells. Incidence is expressed as the fraction of animals showing expression of each growth factor as analyzed by RPA. Mouse (▲), human (■), or mouse/human (●) origin of transcripts was determined using species-specific probes.

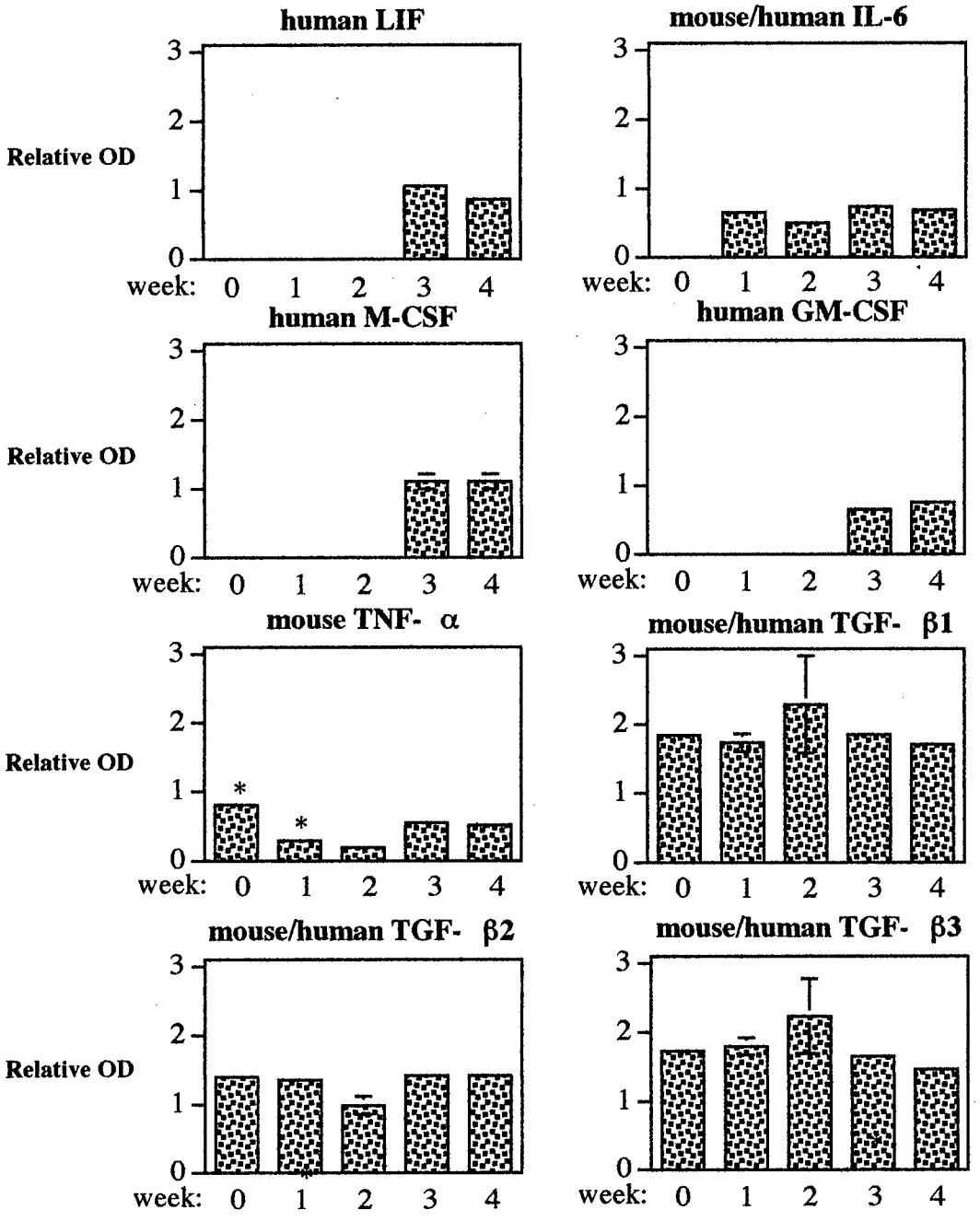


Figure 4. Quantitative analysis of growth factor expression during osteolysis. Bones from nude micewere examined 0, 1, 2, 3, or 4 weeks after cardiac injection with MDA MB 231 breast cancer cells. Quantitative analysis of growth factors expression in mouse bone samples was examined by densitometry of samples analyzed by RPA. Human or mouse origin of transcripts was determined using species-specific probes. *significantly different from week 0, $p<0.05$.

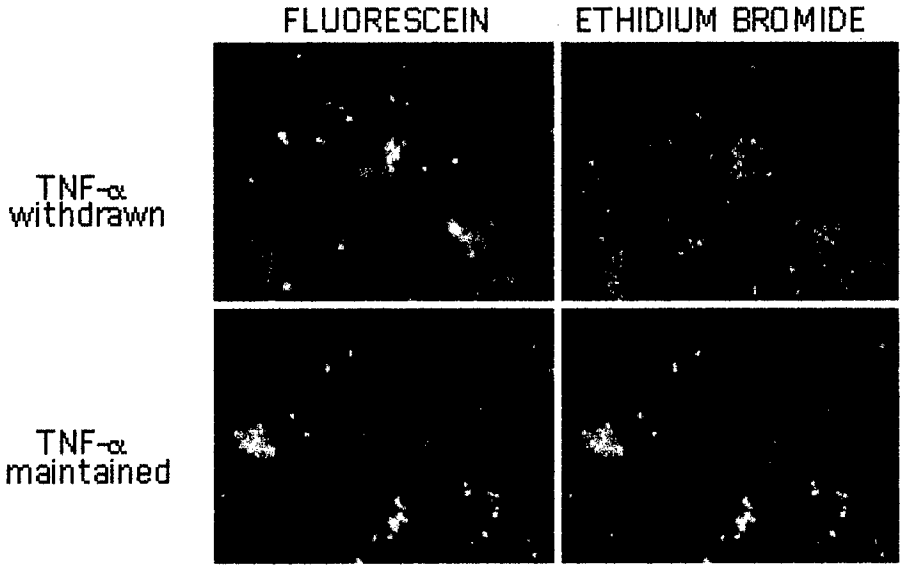
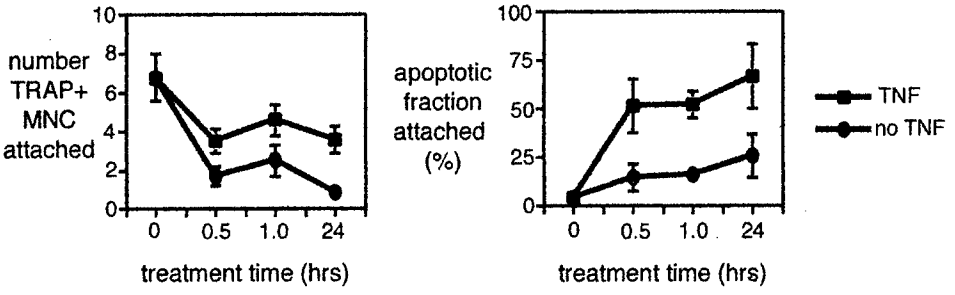


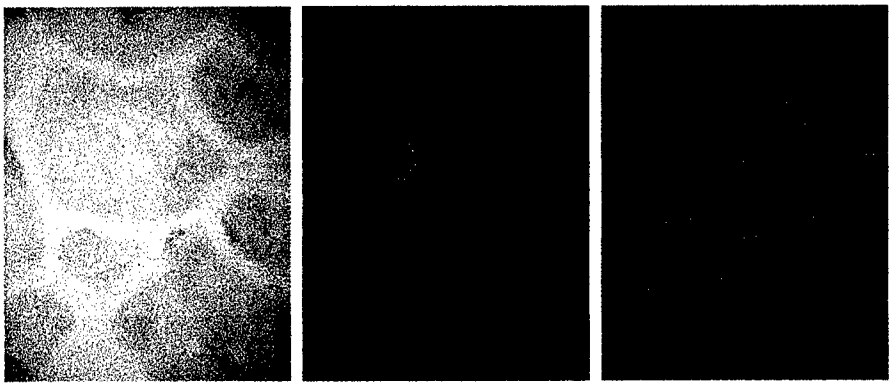
Figure 5. Analysis of cell survival using LIVE/DEAD fluorescent assays: A combination of permeant and impermeant fluorochromes are used to initially assess whether cells are living or dead, as defined by having a functional cell membrane. The LIVE/DEAD kit is used according to manufacturer's instructions to distinguish this category of cells (Molecular Probes). Briefly, cell cultures are incubated in a combination of calcein AM and ethidium homodimer-1, and examined with an epifluorescence microscope equipped with rhodamine and fluorescein filter sets. Esterase activity cleaves calcein AM which traps the green fluorescing calcein (on the left); dying and dead cells become permeable to ethidium homodimer-1 and fluoresce red (on the right). In the top panels, more cells are seen in the right panel than in the left. This indicates more dead than living cells. In the bottom panels, there are no cells positive in the right panel that are not positive in the left panel. This shows that there are fewer apoptotic cells compared with the top panel.

A.



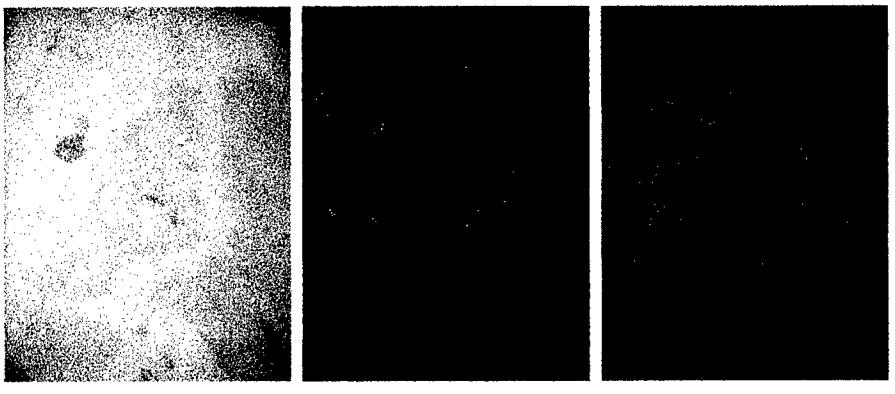
B.

untreated mOCLs



TRAP stain | Hoechst stain | FragEl stain

TNF treated mOCLs



TRAP stain | Hoechst stain | FragEl stain

Figure 6. TNF alpha rapidly induces apoptosis of purified mature osteoclasts. Mature osteoclast-like cells were purified by enzymatic digestion to remove stromal support cells. Cells were then stained with FragEl, Hoechst and TRAP stains and attached mOCLs were counted using visible and epifluorescent microscopy (A). Data are presented as total numbers of TRAP positive multinucleated cells (TRAP+ MNC) or fraction of total MNC that were apoptotic. Micrographs of TRAP, Hoechst and FragEl stained osteoclasts were taken

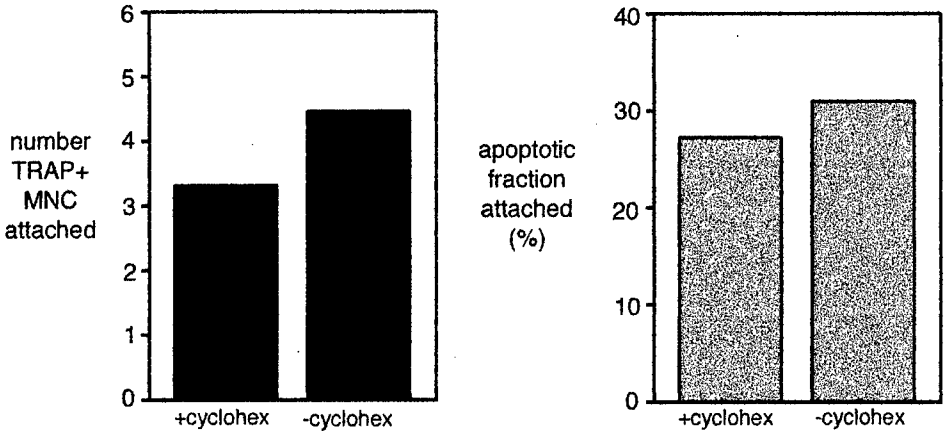


Figure 7. Protein synthesis is not required for TNF-alpha induction of apoptosis. Mature osteoclast-like cells were purified by enzymatic digestion to remove stromal support cells. Cells were treated with 5 mM cycloheximide to block protein synthesis for 90 minutes and then fixed. Cells were then stained with Hoechst and TRAP stains and attached mOCLs were counted using visible and epifluorescent microscopy. Data are presented as total numbers of TRAP positive multinucleated cells (TRAP+ MNC) or fraction of

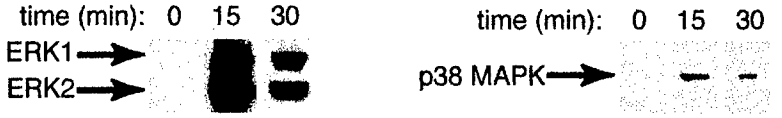


Figure 8A. Purifed osteoclast-like cells rapidly phosphorylate ERK1/2 and p38 MAPK. Mature osteoclast-like cells were purified by enzymatic digestion to remove stromal support cells. Cells were harvested within 0, 15, or 30 minutes after purification and phosphorylation of ERK1/2 and p38 MAK was analyzed by Western immunoblotting using phospho-specific antibodies (New England Biolabs).

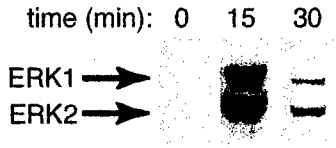


Figure 8B. TNF-alpha suppresses ERK1/2 and p38 MAPK phosphorylation. Mature osteoclast-like cells were purified by enzymatic digestion to remove stromal support cells. Cells were treated with TNF-α and harvested within 0, 15, or 30 minutes after purification. Phosphorylation of ERK1/2 and p38 MAK was analyzed by Western immunoblotting using phospho-specific antibodies (New England Biolabs).

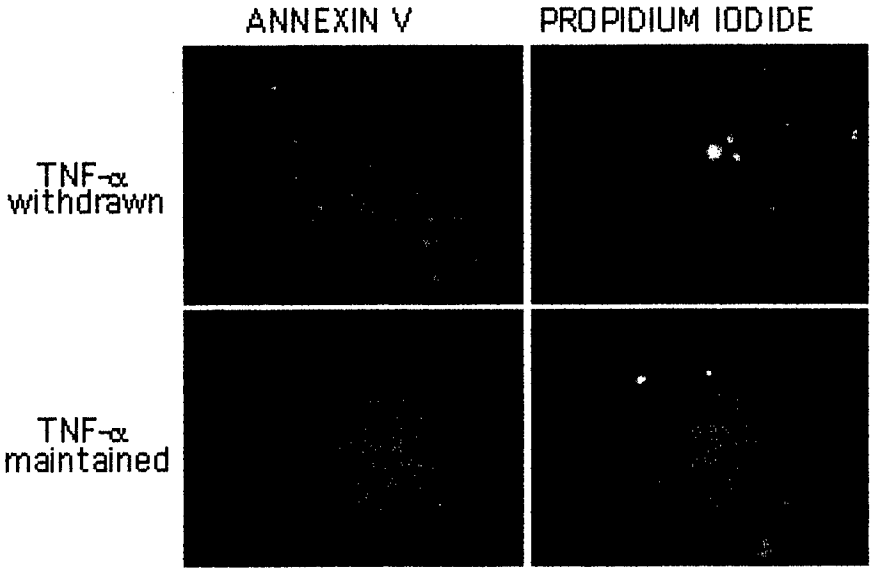


Figure 9. Early Stage Annexin V staining: We are studying early stage of apoptosis by examining alterations in the phospholipid composition of the plasma membrane. Thus, detection of the externalization of phosphatidylserine is a mechanism by which membrane changes during apoptosis are detected. Annexin V binds to phosphatidylserine. Early stages of apoptosis are detected by the use of fluorescently tagged Annexin V to detect externalization of phosphatidylserine. FITC-conjugated Annexin V is used according to manufacturer's instructions (Oncogene Research Products), and membrane fluorescence compared between experimental and control groups. Propidium iodide staining is used to detect mid (cytoplasmic staining) and late (nuclear staining) apoptosis.

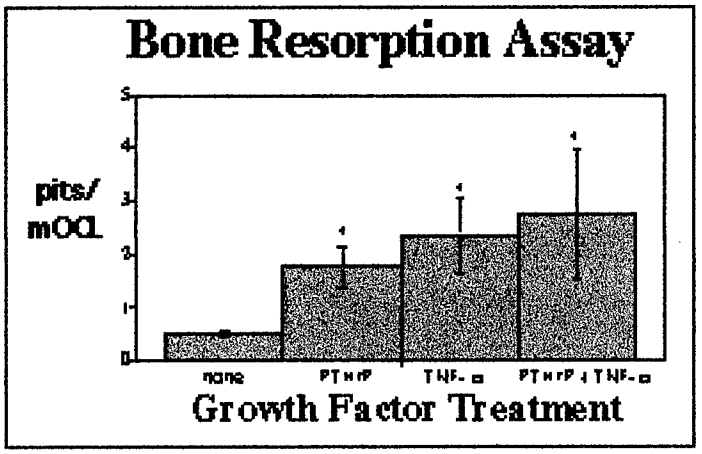


Figure 10. Isolated mouse osteoclast-like cells were generated in the presence of either vehicle or the indicated growth factor (PTHrP: 10 nM, TNF-a: 0.05ng/ml alone or combined), purified, and cultured for 24 hours on bone slices. The level of bone resorption was determined as we have previously described (35).

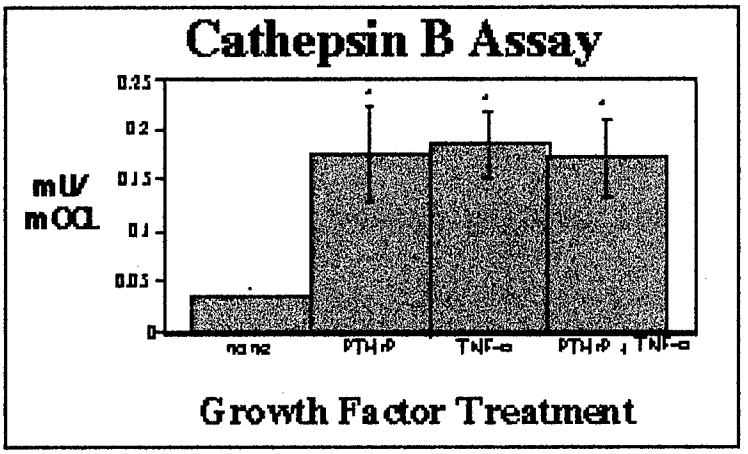


Figure 11. Conditioned media from the above cultures was analyzed for Cathepsin B enzyme activity levels as previously described (36). Activity was normalized to the number of TRAP positive multinucleated cells as determined above.

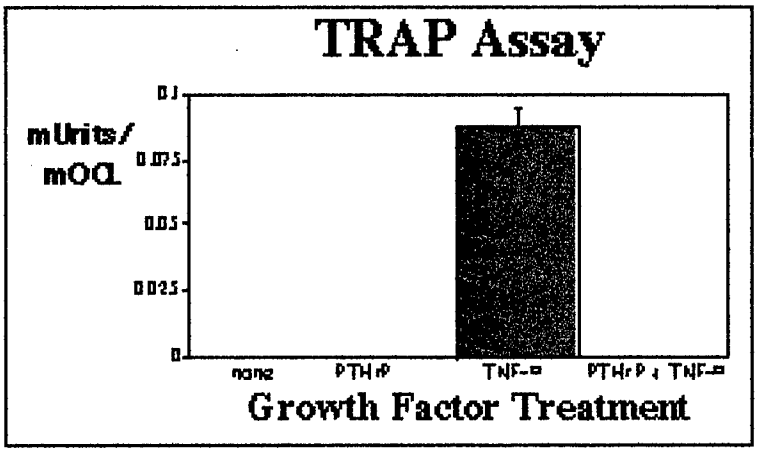


Figure 12. Conditioned media from the above cultures was analyzed for TRAP enzyme activity levels as previously described (36). Activity was normalized to the number of TRAP positive multinucleated cells as determined above.

KEY RESEARCH ACCOMPLISHMENTS:

- determined that breast cancer tumors which have metastasized to bone express GM-CSF, IGF II, TNF-α, and PTHrP mRNAs.
- determined that breast cancer tumors removed from bone secrete IGF-I, IGF II, GM-CSF, PTHrP, and TNF-α.
- TNF-α rapidly induces apoptosis of purified osteoclasts without the requirement of protein synthesis.
- discovered that osteoclasts that differentiate in the presence of TNF-α respond to continued TNF-α exposure with increased survival on the basis of viability and membrane phospholipid changes.
- GM-CSF has no effect on osteoclast lysosomal enzyme secretion whereas IGF II, TNF-α, and PTHrP stimulate osteoclast secretion of cathepsin B and tartrate resistant acid phosphatase.

REPORTABLE OUTCOMES

manuscripts and abstracts presented

1 manuscript submitted to Cancer Research that has been published.

1 review article published

2 manuscripts in preparation for submission

1 abstract to the 1998 American Society for Bone and Mineral Research annual meeting.

1 abstract to the 1999 American Society for Bone and Mineral Research annual meeting.

1 abstract to the 2000 American Society for Bone and Mineral Research annual meeting.

1 abstract and oral presentation 2000 Department of Defense Breast Cancer Research Program Era of Hope Meeting

degress awarded

Aubie Shaw: M.S. in Biology, the University of Minnesota, 2000.

funding applied for:(pending):

Department of the Army: Interactions of RANK Ligand and TNF-α in the Regulation of Osteoclast Survival. June 1 2001 - May 2004 $450,000.

CONCLUSIONS:

We have discovered that breast cancer cells secrete a number of substances that alter osteoclast activity. Our final studies should discover if they work in concert to stimulate bone loss. Of importance is our discovery that tumor cells secrete TNF-α and that osteoclast differentiation in the presence of TNF-α utilize TNF-α as a survival factor. Our data have the potential to impact future therapies as this means that tumor cells stimulate osteoclast differentiation, increase the activity of each osteoclast, and further promote bone loss by extending the life span of each osteoclast. Thus, therapies could be designed to impact any one or more than one of these aspects of stimulation of osteolysis to decrease bone loss.

REFERENCES:

1. Takahashi N, Yamana H, Yoshiki S, Roodman GD, Mundy GR, Jones SJ, Boyde A, Suda T 1988 Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. Endocrinology **122:**1373-1382.
2. Winding B, Misander H, Sveigard C, Therkildsen B, Jakobsen M, Overgaard T, Oursler M, Foged N 2000 Human breast cancer cell-induced angiogenesis, recruitment, and activation of osteoclasts in bone. J Cancer Research and Clinical Oncology **(in press)**.
3. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. Anal Biochem **162**(1):156-9.
4. Miyatake S, Otsuka T, Yokota T, Lee F, Arai K 1985 Structure of the chromosomal gene for granulocyte-macrophage colony stimulating factor: comparison of the mouse and human genes. Embo J **4**(10):2561-8.
5. Shirai T, Shimizu N, Shiojiri S, Horiguchi S, Ito H 1988 Cloning and expression in Escherichia coli of the gene for mouse tumor necrosis factor. Dna **7**(3):193-201.
6. Nedwin GE, Naylor SL, Sakaguchi AY, Smith D, Jarrett-Nedwin J, Pennica D, Goeddel DV, Gray PW 1985 Human lymphotoxin and tumor necrosis factor genes: structure, homology and chromosomal localization. Nucleic Acids Res **13**(17):6361-73.
7. Hattersley G, Chambers T 1990 Effects of interleukin 3 and of granulocyte-macrophage and macrophage colony stimulating factors on osteoclast differentiation from mouse hemopoietic tissue. Journal of Cellular Physiology **142:**201-209.
8. Horwood N, Udagawa N, Elliott J, Grail D, Okamura H, Kurimoto M, Dunn A, Martin T, Gillespie M 1998 Interleukin 18 inhibits osteoclast formation via T cell production of granulocyte macrophage colony-stimulating factor. Journal of Clinical Investigation **101**(3):595-603.
9. Shuto T, Jimi E, Kukita T, Hirata M, Koga T 1994 Granulocyte-macrophage colony stimulating factor suppresses lipopolysaccharide-induced osteoclast-like cell formation in mouse bone marrow cultures. Endocrinology **134**(2):831-837.
10. Udagawa N, Horwood N, Elliott J, Mackay A, Owens J, Okamura H, Kurimoto M, Chambers T, Martin T, Gillespie M 1997 Interleukin-18 (interferon-gamma-inducing factor) is produced by psteoblasts and acts via granulocyte/macrophage colony-stimulating factor and not via interferon-gamma to inhibit osteoclast differentiation. Journal of Experimental Medicine **185**(6):1005-1012.
11. Metcalf D 1986 The molecular biology and functions of the granulocyte-macrophage colony-stimulating factors. Blood **67**(2):257-67.
12. Cecchini MG, Hofstetter W, Halasy J, Wetterwald A, Felix R 1997 Role of CSF-1 in bone and bone marrow development. Mol Reprod Dev **46**(1):75-83; discussion 83-4.
13. Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Morinaga T, Higashio K, Martin TJ, Suda T 2000 Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. J Exp Med **191**(2):275-86.
14. Tashjian AH, Jr., Voelkel EF, Lazzaro M, Goad D, Bosma T, Levine L 1987 Tumor necrosis factor-alpha (cachectin) stimulates bone resorption in mouse calvaria via a prostaglandin-mediated mechanism. Endocrinology **120**(5):2029-36.
15. Li SL, Goko H, Xu ZD, Kimura G, Sun Y, Kawachi MH, Wilson TG, Wilczynski S, Fujita-Yamaguchi Y 1998 Expression of insulin-like growth factor (IGF)-II in human prostate, breast, bladder, and paraganglioma tumors. Cell Tissue Res **291**(3):469-79.
16. Osborne CK, Clemmons DR, Arteaga CL 1990 Regulation of breast cancer growth by insulin-like growth factors. J Steroid Biochem Mol Biol **37**(6):805-9.

17. Hou P, Sato T, Hofstetter W, Foged NT 1997 Identification and characterization of the insulin-like growth factor I receptor in mature rabbit osteoclasts. J Bone Miner Res **12**(4):534-40.
18. Kaji H, Sugimoto T, Kanatani M, Nishiyama K, Nasu M, Chihara K 1997 Insulin-like growth factor-I mediates osteoclast-like cell formation stimulated by parathyroid hormone. Journal of Cellular Physiology **172**(1):55-62.
19. Mochizuki H, Hakeda Y, Wakatsuki N, Usui N, Akashi S, Sato T, Tanaka K, Kumegawa M 1992 Insulin-like growth factor-I supports formation and activation of osteoclasts. Endocrinology **131**:1075.
20. Slootweg M, Most W, van Beek E, Schot L, Papapoulos S, Lowik C 1992 Osteoclast formation together with interleukin-6 production in mouse long bones is increased by insulin-like growth factor-I. Journal of Endocrinology **132**(3):433-438.
21. Nissley SP, Haskell JF, Sasaki N, De Vroede MA, Rechler MM 1985 Insulin-like growth factor receptors. J Cell Sci Suppl **3**:39-51.
22. Neely EK, Beukers MW, Oh Y, Cohen P, Rosenfeld RG 1991 Insulin-like growth factor receptors. Acta Paediatr Scand Suppl **372**:116-23.
23. Blair HC, Teitelbaum SL, Schimke PA, Konsek JD, Koziol CM, Schlesinger PH 1988 Receptor-mediated uptake of a mannose-6-phosphate bearing glycoprotein by isolated chicken osteoclasts. J Cell Physiol **137**(3):476-82.
24. Fiorelli G, Formigli L, Zecchi Orlandini S, Gori F, Falchetti A, Morelli A, Tanini A, Benvenuti S, Brandi M 1996 Characterization and function of the receptor for IGF-I in human preosteoclastic cells. Bone **18**(3):269-276.
25. Middelton J, Arnott N, Walsh S, Beresford J 1995 Osteoblasts and osteoclasts in adult human osteophyte tissue express the mRNAs for insulin-like growth factors I and II and the type I IGF receptor. Bone **16**(3):287-293.
26. Guise TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, Boyce BF, Yoneda T, Mundy GR 1996 Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. J Clin Invest **98**(7):1544-9.
27. Abe E, Tanaka H, Ishimi Y, Miyaura C, Hayashi T, Nagasawa H, Tomida M, Yamaguchi Y, Hozumi M, Suda T 1986 Differentiation-inducing factor purified from conditioned medium of mitogen-treated spleen cell cultures stimulates bone resorption. Proc Natl Acad Sci U S A **83**(16):5958-62.
28. Hilton DJ, Nicola NA, Metcalf D 1991 Distribution and comparison of receptors for leukemia inhibitory factor on murine hemopoietic and hepatic cells. J Cell Physiol **146**(2):207-15.
29. Roodman GD 1992 Interleukin-6: an osteotropic factor? J Bone Miner Res **7**(5):475-8.
30. Ishimi Y, Miyaura C, Jin CH, Akatsu T, Abe E, Nakamura Y, Yamaguchi A, Yoshiki S, Matsuda T, Hirano T, et al. 1990 IL-6 is produced by osteoblasts and induces bone resorption. J Immunol **145**(10):3297-303.
31. Lowik CW, van der Pluijm G, Bloys H, Hoekman K, Bijvoet OL, Aarden LA, Papapoulos SE 1989 Parathyroid hormone (PTH) and PTH-like protein (PLP) stimulate interleukin-6 production by osteogenic cells: a possible role of interleukin-6 in osteoclastogenesis. Biochem Biophys Res Commun **162**(3):1546-52.
32. Shinar CM, Rodan GA 1990 Biphasic Effects of Transforming Growth Factor-B on the Production of Osteoclast-Like Cells in Mouse Bone Marrow Cultures: The Role of Prostaglandins in the Generation of These Cells. Endocrinology **126**:3153-3158.
33. Hattersley G, Chambers TJ 1991 Effects of transforming growth factor beta 1 on the regulation of osteoclastic development and function. J Bone Miner Res **6**(2):165-72.

34. Littlewood-Evans A, Kokubo T, Ishibashi O, Inaoka T, Wlodarski B, Gallagher JA, Bilbe G 1997 Localization of Cathepsin K in Human Osteoclasts by In Situ Hybridization and Immunohistochemistry. Bone **20**(2):81-86.
35. Oursler MJ, Pederson L, Pyfferoen J, Osdoby P, Fitzpatrick L, Spelsberg TC 1993 Estrogen modulation of avian osteoclast lysosomal gene expression. Endocrinology **132**(3):1373-80.
36. Kremer M, Judd J, Rifkin B, Auszmann J, Oursler MJ 1995 Estrogen modulation of osteoclast lysosomal enzyme secretion. J Cell Biochem **57**(2):271-9.

Appendices

Publications:

Shaw, A. and Oursler, M.J. (2000). Paracrine regulation of osteoclast activity in metastatic breast cancer. Cancer Research Alert 1:124-127.

Pederson, L. Winding, B., Foged, N.T., Spelsberg, T.C. and Oursler, M.J. (1999). Identification of breast cancer cell line-derived paracrine factors that stimulate osteoclast activity. Cancer Research 59:5840-5855.

Abstracts:

American Society for Bone and Mineral Research 1998. Metastatic Breast Cancer: Indentification of Factors Involved in Tumor Progression in Bone. A. Shaw, M. Jakobsen, M. Rock, J. Ingle, B. Winding, M.J. Oursler.

American Society for Bone and Mineral Research 1999. TNF-α Stimulates Osteoclast Maturation and Inhibits Apoptosis of Mature Osteoclasts: a Model for Tumor Osteolysis. A. Shaw, A. Gingery, J. Maki, D.A. Pascoe, J. Holy, M.J. Oursler.

American Society for Bone and Mineral Research 2000. TNF-α Rapidly Induces Osteoclast Apoptosis by Direct Activation of Effectors and Suppression of Survival Pathways. A. Gingery, A. Shaw, J. Holy, M.J. Oursler.

Department of Defense Breast Cancer Research Program Era of Hope Meeting, 2000. Identification Of Breast Cancer Cell Line-Derived Paracrine Factors That Increase Osteolysis. A. Gingery, A. Shaw, L. Pederson, B. Winding, N. T. Foged, T. C. Spelsberg, and M. J. Oursler

and prognostic importance of pretreatment clinical characteristics in patients with advanced non-small-cell lung cancer treated with combination chemotherapy. *J Clin Oncol* 1986;4:1604-1614.
12. Takahashi T, Suzuki H, Hida T, et al. The *p53* gene is very frequently mutated in small-cell lung cancer with a distinct nucleotide substitution pattern. *Oncogene* 1991;6:1775-1778.
13. Sameshima Y, Matsuno Y, Hirohashi S, et al. Alterations of the *p53* gene are common and critical events for the maintenance of malignant phenotypes in small-cell lung carcinoma. *Oncogene* 1992;7:451-457.
14. Reichel MB, Ohgaki H, Petersen I, et al. *p53* mutations in primary human lung tumors and their metastases. *Mol Carcinog* 1994;9:105-109.
15. Mitsudomi T, Oyama T, Kusano T, et al. Mutations of the *p53* gene as a predictor of poor prognosis in patients with non-small-cell lung cancer. *J Natl Cancer Inst* 1993;85:2018-2023.
16. Greenblatt MS, Bennett WP, Hollstein M, et al. Mutations in the *p53* tumor suppressor gene: Clues to cancer etiology and molecular pathogenesis. *Cancer Res* 1994;54:4855-4878.
17. D'Amico D, Carbone D, Mitsudomi T, et al. High frequency of somatically acquired *p53* mutations in small-cell lung cancer cell lines and tumors. *Oncogene* 1992;7:339-346.
18. Kure EH, Ryberg D, Hewer A, et al. *p53* mutations in lung tumors: Relationship to gender and lung DNA adduct levels. *Carcinogenesis* 1996;17:2201-2205.
19. Tammemagi MC, McLaughlin JR, Bull SB. Meta-analyses of *p53* tumor suppressor gene alterations and clinicopathological features in resected lung cancers. *Cancer Epidemiol Biomarkers Prev* 1999;8:625-634.
20. Tseng JE, Rodriguez M, Ro J, et al. Gender differences in *p53* mutational status in small cell lung cancer. *Cancer Res* 1999;59:5666-5670.
21. Denissenko MF, Pao A, Tang M, et al. Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in *p53*. *Science* 1996;274:430-432.
22. Eckert KA, Ingle CA, Drinkwater NR. N-ethyl-N-nitrosourea induces A:T to C:G transversion mutations as well as transition mutations in SOS-induced *Escherichia coli*. *Carcinogenesis* 1989;10:2261-2267.
23. Eisenstadt E, Warren AJ, Porter J, et al. Carcinogenic epoxides of benzo[a]pyrene and cyclopenta[cd]pyrene induce base substitutions via specific transversions. *Proc Natl Acad Sci U S A* 1982;79:1945-1949.
24. Ruggeri B, DiRado M, Zhang SY, et al. Benzo[a]-pyrene-induced murine skin tumors exhibit frequent and characteristic G to T mutations in the *p53* gene. *Proc Natl Acad Sci U S A* 1993;90:1013-1017.

Paracrine Regulation of Osteoclast Activity in Metastatic Breast Cancer

By A. Shaw and M. J. Oursler, PhD

BONE IS THE MOST COMMON SITE OF BREAST CANCER metastasis. Breast tumors in the bone marrow cavity recruit osteoclasts to degrade bone, forming localized areas where bone is considerably weakened. Metastatic breast cancer-induced bone loss is termed osteolysis. Osteolysis leads to hypercalcemia and bone fractures, resulting in considerable pain for patients with metastatic breast cancer. Elevated bone resorption by osteoclasts is responsible for tumor-induced osteolysis. The mechanism of the increase in osteoclast activity that causes osteolysis is believed to involve soluble growth factors secreted by the tumor. Recently, the importance of these paracrine influences on osteoclast activity have been recognized, and it is the purpose of this review to discuss the results of investigations into the mechanism of tumor-derived growth factor actions on osteoclasts.

Introduction

Paracrine factors are implicated as mediators of bone loss associated with tumor osteolysis in patients with metastatic breast cancer. Osteolytic lesions result from an increase in osteoclast bone resorption activity at sites adjacent to a tumor in the marrow cavity.[1] Bone loss during tumor osteolysis may result from any of the following mechanisms: 1) increased formation of osteoclasts; 2) increased resorption activity by mature osteoclasts; or 3) increased survival of mature osteoclasts.

Osteolytic bone loss may result from increased formation of mature osteoclasts from osteoclast precursors. Mature osteoclasts form by differentiation from hematopoietic stem cells found in the marrow activity. This process requires direct cell-cell contact between osteoclast precursors and marrow stromal cells. Later stages in osteoclast differentiation include fusion of mononuclear osteoclast precursors to form multinucleated cells, and activation of multinucleated osteoclasts to induce adhesion to bone and secretion of bone-degrading enzymes.[2]

Osteolytic bone loss may result from an increase in the resorption activity of each individual osteoclast. Bone resorption activity is related to the ability of an osteoclast to adhere to bone, secrete bone-degrading lysosomal enzymes, and migrate to form more and/or larger resorption

pits on bone slices. A mature osteoclast may be activated by a number of stimuli. Upon activation, an osteoclast will adhere to bone, forming a tight-sealing zone around the periphery of the bone resorption compartment, which is analogous to a secondary lysosome. Bone-degrading proteases are secreted into this compartment by fusion of lysosomal vesicles with the plasma membrane adjacent to the bone surface. Fusion of these vesicles increases the amount of membrane at this interface and this portion of the plasma membrane becomes highly convoluted, forming a structure called the ruffled border of the osteoclast.

The bone resorption compartment becomes acidified by the action of numerous proton pumps on the ruffled border. The combined action of acid and proteases degrades the bone surface enclosed by the sealing zone. An active osteoclast may detach, migrate, and reattach at another site to form multiple resorption pits. The important role that soluble growth factors play in activation of osteoclasts in metastatic breast cancer was first alluded to by a study that examined the conditioned medium of an osteolytic breast cancer cell line, MDA MB 231.[3]

This study revealed that 231 cell conditioned medium was capable of increasing the activity of mature osteoclasts, suggesting that the medium contained soluble factors that could act on osteoclasts. Analysis of the 231 cell conditioned medium revealed that it contained many growth factors that have been implicated as regulators of osteoclast activity. This conditioned medium was fractionated over a sizing column and used to treat osteoclasts. Some fractions stimulated, while some fractions inhibited osteoclast activity. This observation suggests that stimulatory factors overcome the actions of inhibitory factors to increase osteoclast activity. Paracrine stimulation of osteoclast activity may be a primary mechanism by which metastatic breast tumors are able to degrade bone.

Osteolytic bone loss may result from increased survival of mature osteoclasts. It is believed that mature osteoclasts are removed from the bone surface by a signal to undergo apoptosis. Soluble factors secreted by tumors may delay the apoptotic signal, allowing osteoclasts to continue with their bone resorption program for a longer period of time. Below, several paracrine factors that may be important in the development of osteolytic lesions are discussed.

OPG/RANKL/RANK

Osteoprotegerin (OPG) is a recently discovered protein that plays an important role in osteoclast formation. OPG is a soluble factor secreted by bone marrow stromal cells and osteoblasts. OPG functions as an inhibitor of osteoclast formation. OPG interferes with a critical interaction between the osteoclast precursor receptor-activator of NFkB (RANK) and its cognate ligand (RANKL). RANK is present on the plasma membrane of osteoclast precursors and mature osteoclasts, while RANKL is expressed on the plasma membrane of stromal cells. In addition, the interaction between RANKL and RANK gives rise to signaling events that inhibit apoptosis in mature osteoclasts. In this way, OPG reduces overall osteoclast numbers by blocking osteoclast formation and stimulating apoptosis of mature osteoclasts. Conversely, increased levels of RANKL are correlated with increased osteoclast numbers. One might expect that osteolytic tumors would express RANKL or induce RANKL expression. However, we and others have shown that most primary breast cancers, metastatic breast cancers, and breast cancer cell lines do not express RANKL.[4] When breast cancer cells are co-cultured with bone marrow stromal cells, expression of RANKL is induced and OPG is inhibited.[5] These results indicate that another factor produced by breast cancer cells within the bone marrow cavity induces RANKL expression and inhibits OPG secretion by bone marrow stromal cells.

Macrophage-Colony Stimulating Factor

Macrophage-colony stimulating factor (M-CSF) is absolutely required for osteoclast formation. M-CSF is expressed by bone marrow stromal cells and osteoblasts. The essential role of M-CSF is evident in mutant op/op mice that express nonfunctional M-CSF. These mice show a complete lack of mature osteoclasts, a condition that is reversed by infusion of M-CSF. M-CSF stimulates proliferation of early osteoclast precursors and induces migration of actively resorbing osteoclasts. Estrogen deficiency enhances M-CSF production, which results in the overall increase in osteoclast number seen in osteoporosis. M-CSF also promotes the survival of mature osteoclasts by delaying the onset of apoptosis. M-CSF and RANKL have been shown to be essential factors for osteoclast formation in vitro.[6]

Granulocyte-Macrophage Colony Stimulating Factor

Granulocyte-macrophage colony stimulating factor (GM-CSF) has opposing effects on osteoclast formation. Effects of GM-CSF appear to depend on the differentiation state of the target cell. GM-CSF stimulates proliferation of early osteoclast precursors, but potently inhibits late stages of osteoclast differentiation. GM-CSF inhibits formation of osteoclasts from mouse bone marrow, which contains mid-stage osteoclast precursors. In an in vivo nude mouse model of osteolysis, expression of GM-CSF declined as osteolytic lesions appeared.[7] Local repression of GM-CSF to allow osteoclast formation may provide a mechanism by which an osteolytic tumor can mediate bone loss.

Tumor Necrosis Factor Alpha

Tumor necrosis factor alpha (TNF-α) is a soluble protein secreted by many bone marrow cells and is known to play a role in the development of other bone loss pathologies, including periodontitis and orthopedic implant loosening. Antibody blockade of TNF-α results in decreased osteoclast numbers and reduced pit formation activity of mature osteoclasts.[8] TNF-α is secreted in large quantities by osteolytic breast cancer cell lines and by breast tumors in bone. We have used a mouse model of breast cancer osteolysis to examine the timing of TNF-α expression as it relates to the appearance of osteolytic lesions. TNF-α expression by mouse marrow cells increases as tumor size increases and as osteolytic lesions appear in the mice. We have examined the effects of TNF-α added to osteoclast precursors during differentiation. TNF-α treatment results in increased osteoclast numbers and larger osteoclasts as compared to untreated osteoclasts. These larger TNF-α-stimulated osteoclasts secrete more bone-degrading enzymes and form more resorption pits per cell than unstimulated osteoclasts. TNF-α also appears to prolong the lifespan of mature osteoclasts by inhibiting apoptosis.[9] These results indicate that elevated TNF-α levels may play a role in metastatic breast cancer-induced osteolysis. TNF-α secreted by tumor cells and marrow cells may induce formation of larger osteoclasts that destroy bone at a rate faster than it can be replaced by osteoblasts.

Insulin-Like Growth Factors

Insulin-like growth factors (IGFs) are secreted by osteoblasts and stimulate proliferation of osteoblasts. Mature osteoclasts express type I IGF receptors and IGF stimulates resorption activity of mature osteoclasts in the presence of osteoblasts.[10] IGF induction is responsible for growth hormone and parathyroid hormone-induced osteoclast formation.[11] This suggests that IGF may contribute to osteoclast formation indirectly by inducing expression of another osteoclast-promoting growth factor. IGF is secreted by breast cancer cell lines and metastatic breast tumors in bone. IGF treatment of osteoclast precursors induces differentiation into mature osteoclasts. IGF secreted by metastatic breast tumors may contribute to elevated osteoclast activity by inducing differentiation of osteoclast precursors and stimulating resorption activity of mature osteoclasts.

Parathyroid Hormone-Related Peptide Expression

Parathyroid hormone-related peptide (PTHrP) expression is correlated with increased metastasis of breast cancer cells, which leads to increased frequency of osteolytic lesions in vivo. PTHrP is expressed by mature osteoclasts and 92% of breast tumors showing bone metastases.[12] PTHrP is the main causative agent of hypercalcemia that is associated with a variety of cancers. Hypercalcemia is mediated through an endocrine mechanism involving PTH receptor-mediated effects on kidney and bone metabolism. Paracrine actions of PTHrP are dual, with different portions of the PTHrP molecule giving rise to opposing effects. PTHrP(1-34) mediates stimulatory PTH-like actions by binding to the PTH receptor expressed on osteoblasts. These indirect effects include stimulation of osteoclast formation and increased bone resorption activity. However, PTHrP(107-139) mediates inhibitory actions of PTHrP, including direct inhibition of osteoclastic bone resorption, by an unknown PTH receptor-independent mechanism.[13] PTHrP expression appears to be permissive for formation of bone metastases, although its role in the induction of osteolysis remains unclear.

Interleukins

Interleukin-1 (IL-1) stimulates bone resorption primarily by prolonging survival of mature osteoclasts. IL-6 also stimulates bone resorption, but its mode of action is stimulation of osteoclast formation. IL-6 induces proliferation of osteoclast precursors and induces these precursors to commit to the osteoclast lineage. Infusion of IL-1 together with IL-6 in mice causes marked bone loss that results in hypercalcemia. Bone loss due to elevated levels of parathyroid hormone or 1, 25-dihydroxyvitamin D3 is due to the ability of these hormones to induce IL-6 secretion from osteoblasts.[6] Many breast tumors express IL-1 and IL-6, which makes these factors possible mediators of bone loss associated with metastatic breast cancer.

Leukemia Inhibitory Factor

Leukemia inhibitory factor (LIF) is so named because of its ability to inhibit proliferation and induce differentiation to the macrophage line of a myeloid leukemic cell line.[14] Since its discovery, a variety of systemic effects have been attributed to LIF. The overall effect of LIF on bone metabolism is to increase the rate of bone turnover by increasing both osteoblast and osteoclast activity. LIF overexpression in mice causes splenic enlargement due to the excessive proliferation of hematopoietic stem cells, the cells that give rise to osteoclasts. LIF prolongs survival of osteoclast precursors and induces the proliferation of osteoblasts. LIF expression is induced by cytokines known to induce bone loss, including TNF-α, IL-1, and IL-6.[15] LIF is expressed by 78% of primary breast tumors and stimulates the proliferation of breast cancer cell lines in vitro.[16] Expression of LIF by breast cancers in bone could potentially stimulate osteoclast activity to induce bone loss.

Transforming Growth Factor

Transforming growth factor (TGF-β) has biphasic effects on osteoclast activity. At high doses, TGF-β inhibits osteoclast formation. However, at low doses, TGF-β stimulates formation and survival of osteoclasts. We have shown that osteoclasts formed in the presence of TGF-β become TGF-β-dependent. Withdrawal of TGF-β from these TGF-β-dependent osteoclasts induces immediate apoptosis.[17] Osteoclasts formed in the environment adjacent to a TGF-β-secreting breast tumor may become TGF-β-dependent and may survive longer than osteoclasts formed in the absence of TGF-β. Enhanced survival of TGF-β-dependent osteoclasts may provide a mechanism by which breast tumors can induce osteolysis.

Summary

Breast tumors in the bone marrow cavity can induce bone loss by inducing the formation, activity, and survival of mature osteoclasts. These effects may be due to the secretion of growth factors in the area adjacent to the tumor. RANKL, M-CSF, TNF-α, IGF, IL-1, IL-6, LIF, and TGF-β can enhance formation of mature osteoclasts by exerting effects on various stages of osteoclast differentiation. RANKL, M-CSF, TNF-α, IGF, and LIF can promote osteoclast resorption activity by increasing adhesion to bone, secretion of lysosomal proteases, and migration. M-CSF, TNF-α, IL-1, and TGF-β increase the lifespan of mature osteoclasts by delaying apoptosis. Additionally, repression of factors that block osteoclast formation, such as GM-CSF, may enable tumors to recruit osteoclasts. In order to clarify the role of tumor-derived growth factors in development of osteolytic lesions, future investigations should explore the combined effects of growth factors on osteoclast activity. *(Ms. Shaw is a research assistant and Dr. Oursler is an Assistant Professor, Biology Department, University of Minnesota, Duluth.)* ❖

References

1. Clohisy DR, Palkert D, Ramnaraine MLR, et al. Human breast cancer induces osteoclast activation and increases the number of osteoclasts at sites of tumor osteolysis. *J Bone Joint Surg* 1996;14:396-402.
2. Reddy S, Roodman GD. Control of osteoclast differentiation. *Crit Rev Euk Gene Exp* 1998;8:1-17.
3. Pederson L, Winding B, Foged N, et al. Identification of breast cancer cell line-derived paracrine factors that stimulate osteoclast activity. *Cancer Res* 1999;59: 5849-5855.
4. Thomas R, Guise T, Yin J, et al. Breast cancer cells interact with osteoblasts to support osteoclast formation. *Endocrinol* 1999;140:4451-4458.
5. Chikatsu N, Takeuchi Y, Tamura Y, et al. Interactions between cancer and bone marrow cells induce osteoclast differentiation factor expression and osteoclast-like cell formation in vitro. *Biochem Biophys Res Commun* 2000;67:632-637.
6. Martin TJ, Romas E, Gillespie M. Interleukins in the control of osteoclast differentiation. *Crit Rev Euk Gene Exp* 1998;8:107-123.
7. Shaw A, Jakobsen M, Rock M, et al. *Metastatic breast cancer: Identification of factors involved in tumor progression in bone.* In: Second Joint Meeting of the American Society for Bone and Mineral Research and the International Bone and Mineral Society, San Francisco, CA; November 1998:S280.
8. Tani-Ishii N, Tsunoda A, Teranaka T, et al. Autocrine regulation of osteoclast formation and bone resorption by IL-1 alpha and TNF alpha. *J Dent Res* 1999;78: 1610-1623.
9. Shaw A, Gingery A, Maki J, et al. *TNF alpha stimulates osteoclast maturation and inhibits apoptosis of mature osteoclasts: A model for tumor osteolysis.* In: 21st Annual Meeting of the American Society for Bone and Mineral Research: St. Louis, MO; September 1999:S484.
10. Conover C. The role of insulin-like growth factors and binding proteins in bone cell biology. *Principles of Bone Biology*. Academic Press; 1996:607-618.
11. Chihara K, Sugimoto T. The action of GH/IGF-I/IGFBP in osteoblasts and osteoclasts. *Horm Res* 1997;48: 45-49.
12. Powell G, Southby J, Danks J, et al. Localization of parathyroid hormone-related protein in breast cancer metastases: Increased evidence in bone compared with other sites. *Cancer Res* 1991;51:3059-3061.
13. Rankin W, Grill V, Martin TJ. Parathyroid hormone-related protein and hypercalcemia. *Cancer* 1997;80: 1564-1571.
14. Van Beek E, Van der Wee-Pals L, Van de Ruit M, et al. Leukemia inhibitory factor inhibits osteoclastic resorption, growth, mineralization, and alkaline phosphatase activity in fetal mouse metacarpal bones in culture. *J Bone Miner Res* 1993;8:191-198.
15. Kurzrock R, Estrov Z, Wetzler M, et al. LIF: Not just a leukemia inhibitory factor. *Endocrine Rev* 1991;12: 208-217.
16. Dhingra K, Sahin A, Emami K, et al. Expression of leukemia inhibitory factor and its receptor in breast cancer: A potential autocrine and paracrine growth regulatory mechanism. *Breast Cancer Res Treat* 1998;48:165-174.
17. Gingery A, Shaw A, Holy J, et al. *TGF beta is a survival factor for TGF beta-induced osteoclasts.* In: 21st Annual Meeting of the American Society for Bone and Mineral Research: St. Louis, MO; September 1999:S300.

[CANCER RESEARCH 59, 5849–5855, November 15, 1999]

Identification of Breast Cancer Cell Line-derived Paracrine Factors That Stimulate Osteoclast Activity[1]

Larry Pederson, Bent Winding, Niels T. Foged, Thomas C. Spelsberg, and Merry Jo Oursler[2]

Department of Biochemistry and Molecular Biology, Mayo Clinic and Foundation, Rochester, Minnesota 55905 [L. P., T. C. S.]; Center for Clinical and Basic Research, Ballerup, Byvei 222 DK-2750, Ballerup, Denmark [B. W., N. T. F.]; and Departments of Biology, Medical Microbiology and Immunology, and Biochemistry and Molecular Biology, University of Minnesota, Duluth Minnesota, 55812 [M. J. O.]

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 irradicate 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 bone, 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 1,[3] 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% CO_2 until confluent. Cell monolayers were washed with sterile PBS (pH 7.4) and placed in αMEM

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

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Department of the Army Grant DAMD17-1-7157, NIH Grant R21 CA 65800 to the Mayo Clinic Cancer Center, and The Whiteside Institute for Clinical Research.

[2] To whom requests for reprints should be addressed, at University of Minnesota, Duluth Campus, Department of Biology, 211 Life Science Building, 10 University Drive, Duluth, MN 55812-2496.

[3] 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.

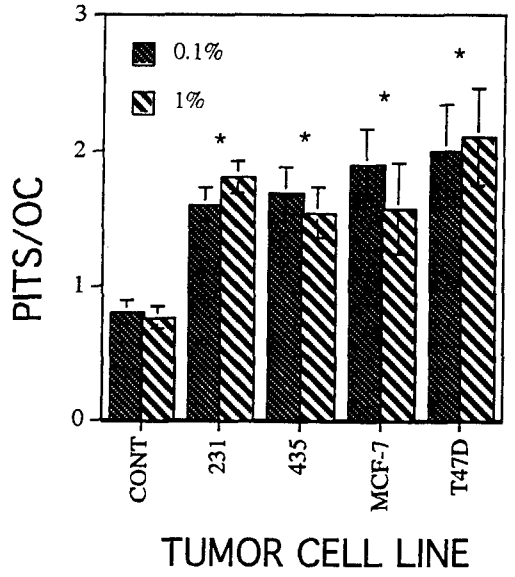


Fig. 1. Osteoclast responses to selected breast cancer cell line-conditioned media. Isolated osteoclasts were cultured with the indicated concentration of the following tumor cell line-conditioned media: MDA MB 231 (*231*), MDA MB 435 (*435*), MCF-7, and T47D, or control (*CONT*). Analysis was done after 24 h of culture. The number of pits formed per osteoclast per 1-mm^2 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; *, $P < 0.05$.

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°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-mm^2 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 mm^2 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).

Lysosomal 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 *o*-carboxy phenyl phosphate was determined by following the

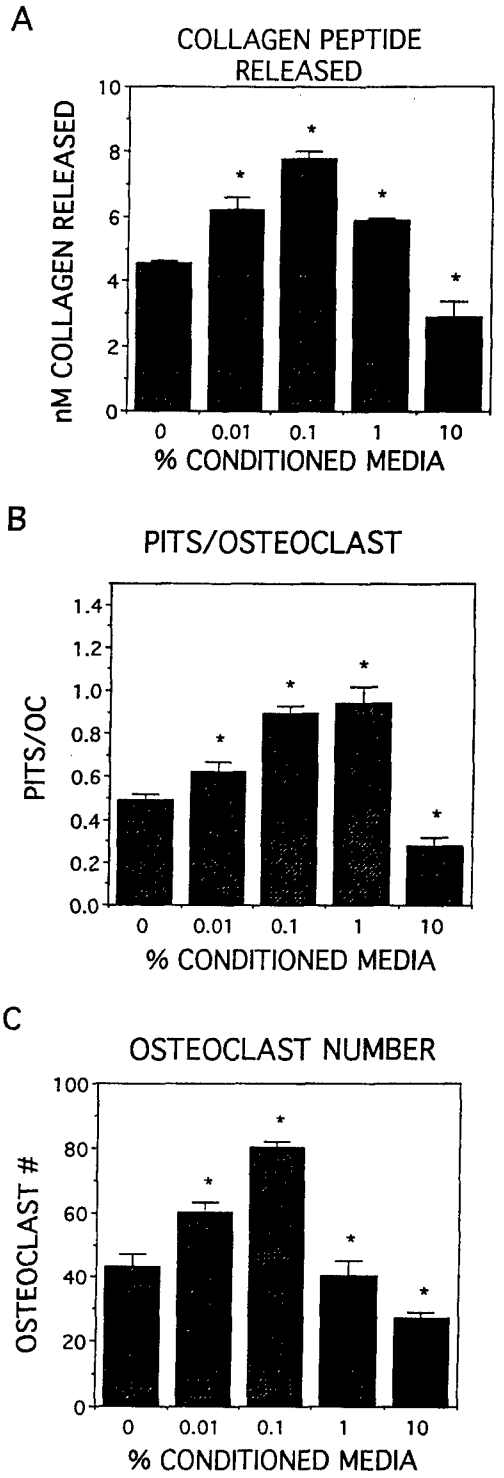


Fig. 2. Effects of human breast cancer cell line-conditioned media on osteoclast resorption activity. Isolated osteoclasts were cultured with the indicated concentration of MDA MB 231 tumor cell line-conditioned media. Analysis was done after 24 h of culture. *A*, the amount of collagen peptide released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

Table 1 *Growth factor/cytokine analysis of conditioned media*

Conditioned media from MDA MB 231 cells were analyzed in triplicate for the indicated growth factors. Analysis was performed from three separate batches of conditioned media, and these results are representative of these analyses. The results are the mean ± SE of the replicates.

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

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. 2*A*). 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

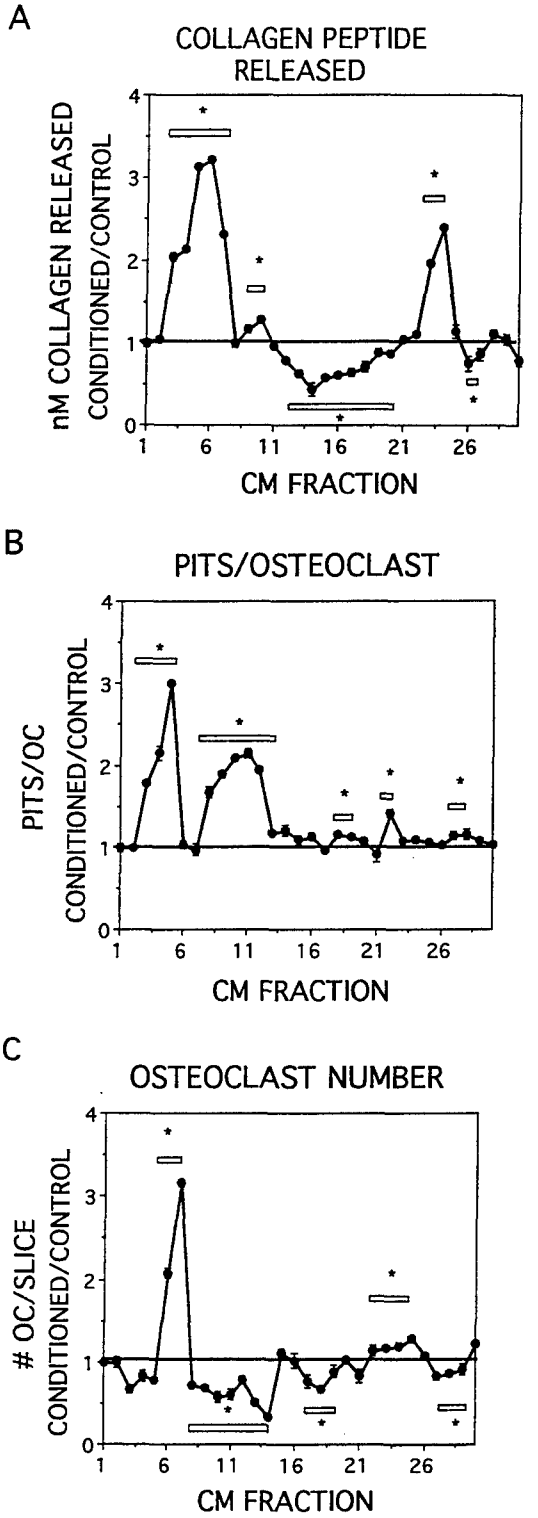


Fig. 3. Size fractionation of MDA MB 231-conditioned media. Conditioned media (0.5 ml) was fractionated and added to aliquots of freshly isolated osteoclasts on slices of bone after filter sterilization. Analysis was done after 24 h of culture. Results are presented for *A*, the amount of collagen peptide released into the media; *B*, the number of pits formed per osteoclast per 1-mm^2 bone slice; *C*, the number of osteoclasts per 1-mm^2 bone slice. Each experiment was done in triplicate, and the results are presented as the mean ± SE; *, $P < 0.001$.

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.

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

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. 2*B*). 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. 2*C*). 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. 3*A*). 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. 3*B*). 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. 3*C*). 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 assayed 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-

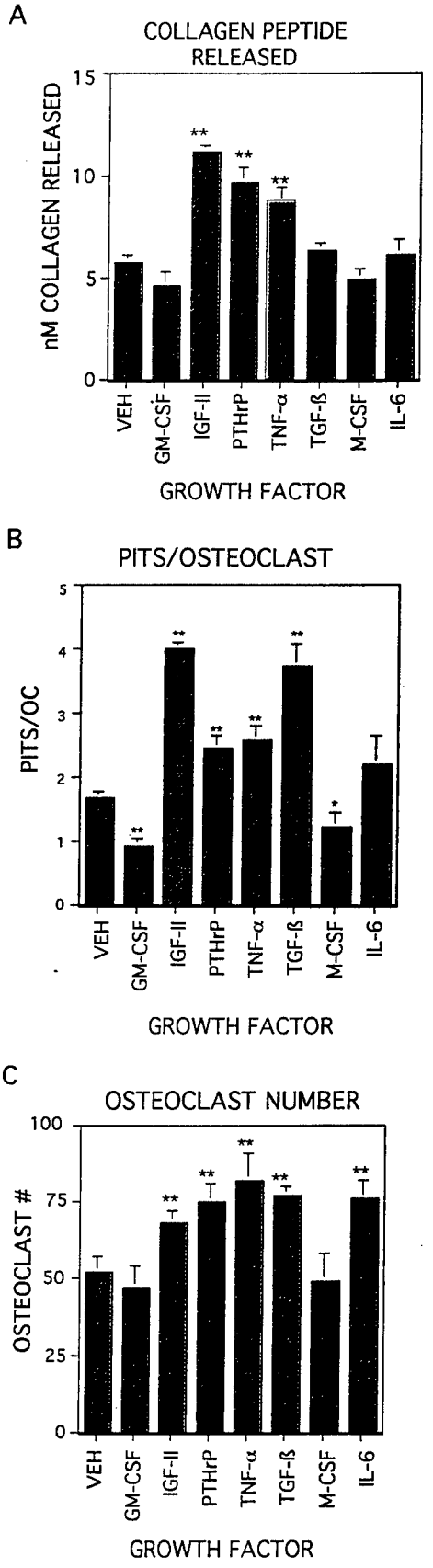


Fig. 4. Analysis of selected factor effects on bone resorption activity. Isolated osteoclasts were cultured with either vehicle (media with 0.25% BSA) or the indicated factors at the following concentrations for 24 h: GM-CSF, 0.15 ng/ml; IGF-II, 10 pg/ml; PTHrP, 5 ng/ml; TNF-α, 15 pg/ml; TGF-β_2, 50 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-mm^2 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.

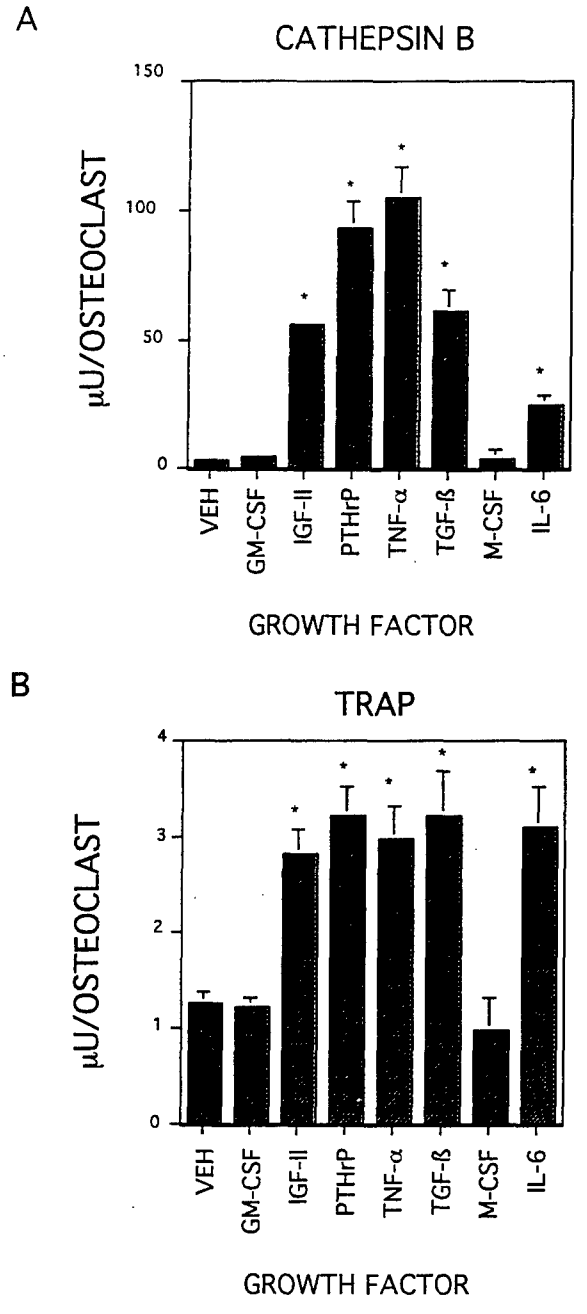


Fig. 5. Analysis of selected factor effects on lysosomal enzyme secretion. Isolated osteoclasts were cultured with either vehicle (media with 0.25% BSA) or the indicated factors at the following concentrations for 24 h: GM-CSF, 0.15 ng/ml; IGF-II, 10 pg/ml; PTHrP, 5 ng/ml; TNF-α, 15 pg/ml; TGF-β_2, 50 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*, cathepsin B activity in the conditioned media and *B*, TRAP activity in the conditioned media. Assays were performed on seven slices/treatment. The experiment was done a total of three times, and these are representative results. Results are mean ± SE; *, $P < 0.01$.

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 cathepsin 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. Quantitation 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 fractions were in midrange (estimated sizing between M_r 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

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

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 + M-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.

Treatment	Collagen nM	Pits/OC[a]	No. of OC	CATH B	TRAP
Vehicle	1.7 ± 0.2	0.46 ± 0.09	43 ± 7	34 ± 9	1.5 ± 0.7
0.1% conditioned media	9.2 ± 0.4[b]	0.87 ± 0.10[c]	88 ± 12[c]	299 ± 26[b]	8.2 ± 0.4[b]
IGF-II + TGF-β + PTHrP + TNF-α	9.7 ± 0.5[b]	0.91 ± 0.14[c]	103 ± 4[b]	258 ± 27[b]	8.6 ± 0.4[b]
GM-CSF + M-CSF + IL-6 + PTHrP + TGF-β	0.4 ± 0.4[c]	0.27 ± 0.07[c]	39 ± 9	12 ± 8[c]	0.8 ± 0.2
TNF-α + IGF-II + GM-CSF + M-CSF + IL-6 + PTHrP + TGF-β	5.1 ± 0.3[b]	0.36 ± 0.09[c]	55 ± 7[b]	87 ± 16[b]	2.3 ± 0.3[b]

[a] OC, osteoclast; CATH B, cathepsin B.
[b] $P < 0.001$ comparing vehicle to treatment.
[c] $P < 0.01$ comparing vehicle to treatment.

number of osteoclasts per slice was decreased; thus, the stimulation in resorption activity is likely to be due to the elevation in activity of the individual osteoclast. In contrast, fractions 6 and 7 significantly stimulated the number of cells per slice, whereas there was no significant effect on the activity per osteoclast. Thus, the elevation in the amount of total bone excavated is likely to be, at least in part, the result of an increase in the number of cells on the bone slices, counterbalancing the lack of any effect on the activity per osteoclast. Because fraction 6 contained TNF-α, we examined the effects of TNF-α on osteoclast numbers. The data presented here supports the theory that TNF-α elevates the number of osteoclasts found on bone after short-term treatment, and we are presently pursuing the mechanisms of this effect. Fractions 9 and 10 caused an increase in the activity of each osteoclast, whereas the number of osteoclasts per slice was decreased. These combined to slightly stimulate bone resorption. Fraction 10 contains IGF-II, and our data demonstrate that the major influence of IGF-II is on the activity of individual osteoclasts, supporting the theory that IGF-II may be important in tumor-driven stimulation of osteoclast activity. When the amount of collagen peptide released was examined, fractions 23 and 24 stimulated bone resorption. In these fractions, there was no effect observed on the activity level of the osteoclasts and very small stimulation in the number of osteoclasts present. It may be that the elevation in collagen peptide released in these samples was due to each osteoclast generating a larger resorption pit. Interestingly, fractions 11 through 20 inhibited the amount of bone removed. In these fractions, the activity of each osteoclast was stimulated, whereas the number of osteoclasts was depressed. There are many growth factors present in these fractions, and our data demonstrate that the interactions of these growth factors results in repressed bone resorption and lysosomal enzyme secretion. The observed decrease in total bone loss may be the result of a decrease in osteoclast binding, an elevation in apoptosis, or may also be due to shallower pits being generated. The precise nature of this observation and the interactions of these growth factors will also require further study. This effect of repressing resorption by these fractions may at least in part explain the biphasic nature of the dilution curve observed above. If substances are present in the media that repress resorption, the higher concentrations of them could repress activity despite the presence of the stimulatory agents.

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 finding, 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 knockouts seem 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 4 *Comparative analysis of conditioned media fractions*

With reference to the fractions numbers indicated on the left, the effects of treatment with the conditioned media in the indicated fraction on the total volume of bone removed (obtained by collagen peptide release measurement), the activity per cell (obtained from the pits/osteoclast measurement), and the osteoclast number (obtained from the osteoclast per slice measurement) provide some indication of the predominant effect of the conditioned media fraction on bone resorption. Assuming that the amount of bone removed indicates the overall effect on bone resorption activity, it is possible to project the likely regulatory effects as indicated in the last column on the right.

Fraction no.	Total volume of bone removed	ACT[a]/cell	OC no.	Regulatory factor causing response
3–5	↑	↑	↓	↑ OC ACT/cell
6, 7	↑	↔	↑	↑ No. of OCs bound
9, 10	slight ↑	↑	↓	↑ Counter balance OC ACT/cell and no. of OCs bound
11–20	↓	variable ↑	variable ↓	↓ No. of OC bound ? Pit volume
23, 24	↑	↔	slight ↑	↑ ? Pit volume

[a] 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.

ACKNOWLEDGMENTS

The tireless work of Ryan Sportel in the resorption and lysosomal enzyme assays is gratefully acknowledged.

REFERENCES

1. Dickson, R. B., and Lippman, M. E. Growth factors in breast cancer. Endocr. Rev., *16:* 559–589, 1995.
2. Guise, T. A., and Mundy, G. R. Cancer and bone. Endocr. Rev., *19:* 18–54, 1998.
3. Roodman, G. D. Advances in bone biology: the osteoclast. Endocr. Rev., *17:* 308–332, 1996.
4. Suda, T., Nakamura, I., Jimi, E., and Takahashi, N. Regulation of osteoclast function. J. Bone Miner. Res., *12:* 869–879, 1997.
5. Gao, Y., Morita, I., Maruo, N., Kubota, T., Murota, S., and Aso, T. Expression of IL-6 receptor and GP 130 in mouse bone marrow cells during osteoclast differentiation. Bone (N.Y.), *22:* 487–493, 1998.
6. Hofstetter, W., Wetterwald, A., Cecchini, M., Felix, R., Fleisch, H., and Mueller, C. Detection of transcripts for the receptor for macrophage colony-stimulating factor, c-fms, in murine osteoclasts. Proc. Natl. Acad. Sci. USA, *89:* 9637–9641, 1992.
7. Hou, P., Sato, T., Hofstetter, W., and Foged, N. Identification and characterization of the insulin-like growth factor I receptor in mature rabbit osteoclasts. J. Bone Miner. Res., *12:* 534–540, 1997.
8. May, L. G., and Gay, C. V. Parathyroid hormone uses both adenylate cyclase and protein kinase C to regulate acid production in osteoclasts. J. Cell. Biochem., *65:* 565–573, 1997.
9. Sunyer, T., Lewis, J., Collin-Osdoby, P., and Osdoby, P. Estrogen's bone-protective effects may involve differential IL-1 receptor regulation in human osteoclast-like cells. J. Clin. Invest., *103:* 1409–1418, 1999.
10. Zheng, M. H., Fan, Y., Wysocki, S. J., Lau, A. T., Robertson, T., Beilharz, M., Wood, D. J., and Papadimitriou, J. M. Gene expression of transforming growth factor-β1 and its type II receptor in giant cell tumors of bone. Possible involvement in osteoclast-like cell migration. Am. J. Pathol., *145:* 1095–1104, 1994.
11. Oursler, M., Collin-Osdoby, P., Anderson, F., Li, L., Webber, D., and Osdoby, P. Isolation of avian osteoclasts: improved techniques to preferentially purify viable cells. J. Bone Miner. Res., *6:* 375–385, 1991.
12. Collin-Osdoby, P., Oursler, M. J., Webber, D., and Osdoby, P. Osteoclast-specific monoclonal antibodies coupled to magnetic beads provide a rapid and efficient method of purifying avian osteoclasts. J. Bone Miner. Res., *6:* 1353–1365, 1991.
13. Oursler, M. J., Pederson, L., Pyfferoen, J., Psdpby, P., Fitzpatrick, L., and Spelsberg, T. C. Estrogen modlulation of avian osteoclast lysosomal gene expression. Endocrinology, *132:* 1373–1380, 1993.
14. Oursler, M. J., Pederson, L., Fitzpatrick, L., Riggs, B. L., and Spelsberg, T. C. Human giant cell tumors of the bone (osteoclastomas) are estrogen target cells, Proc. Natl. Acad. Sci. USA, *91:* 5227–5231, 1994.
15. Foged, N. T., Delaisse, J-M., Hou, P., Lou, H., Sato, T., Winding, B., and Bonde, M. Quantification of the collageonlytic activity of isolated osteoclasts by enzyme-linked immunosorbent assay. J. Bone Miner. Res., *11:* 226–237, 1996.
16. Hofstee, B. H. J. Direct and continuous spectrophotometric assay of phosphomonoesterases. Arch. Biochem. Biophys., *51:* 139–144, 1954.
17. Barrett, A., and Kirschke, H. Proteases of diverse origin and functin, cathepsin B, cathepsin D, cathepsin H., and cathepsin L. Methods Enzymol., *80:* 535–561, 1981.
18. De Leon, D. D., and Asmerom, Y. Quantification of insulin-like growth factor I without interference by IGF binding proteins. Endocrinology, *138:* 2199–2202, 1997.
19. Nakai, M., Mundy, G. R., Williams, P. J., Boyce, B., and Yoneda, T. A. A synthetic antagonist to laminin inhibits the formation of osteolytic metastases by human melanoma cells in nude mice. Cancer Res., *52:* 5395–5399, 1992.
20. Chihara, K., and Sugimoto, T. The action of GH/IGF-I/IGFBP in osteoblasts and osteoclasts. Horm. Res. (Basel), *48:* 45–49, 1997.
21. Hill, P., Reynolds, J., and Meikle, M. Osteoblasts mediate insulin-like growth factor-I and -II stimulation of osteoclast formation and function. Endocrinology, *136:* 124–131, 1995.
22. Vargas, S., Naprta, A., Glaccum, M., Lee, S., Kalinowski, J., and Lorenzo, J. Interleukin-6 expression and histomorphometry of bones from mice deficient in receptors for interleukin-1 or tumor necrosis factor. J. Bone Miner. Res., *11:* 1736–1744, 1996.
23. Jimi, E., Shuto, T., and Koga, T. Macrophage colony-stimulating factor and interleukin-1 α maintain the survival of osteoclast-like cells. Endocrinology, *136:* 808–811, 1995.
24. Manolagas, S. C. Role of cytokines in bone resorption. Bone (N.Y.), *17:* 63S–67S, 1995.
25. Adebanjo, O., Moonga, B., Yamate, T., Sun, L., Minkin, C., Abe, E., and Zaidi, M. Mode of action of interleukin-6 on mature osteoclasts. Novel interactions with extracellular Ca^{2+} sensing in the regulation of osteoclastic bone resorption. J. Cell Biol., *142:* 1347–1356, 1998.
26. Kuroda, H., Nakamura, M., and Kamiyama, K. Effects of calcitonin and parathyroid hormone on the distribution of F-actin in the clear zone of osteoclasts *in vivo*. Bone (N.Y.), *19:* 115–120, 1996.
27. Dieudonne, S., Foo, P., Van Zoelen, E., and Burger, E. Inhibiting and stimulating effects of TGF-B_1 on osteoclastic bone resorption in fetal mouse bone organ cultures. J. Bone Miner. Res., *6:* 479–487, 1991.
28. Hughes, D. E., Wright, K. R., Mundy, G. R., and Boyce, B. F. $TGFB_1$ induces osteoclast apoptosis *in vitro*. J. Bone Miner. Res., *9:* S138, 1994.

ABSTRACT American Society for Bone and Mineral Research 1998

Metastatic Breast Cancer: Identification Of Factors Involved In Tumor Progression In Bone. A. Shaw*, M. Jakobsen*, M. Rock*, J. Ingle*, B. Winding, M.J. Oursler. University of Minnesota, Duluth, MN; Center for Clinical and Basic Research, Ballerup, Denmark; Mayo Clinic, Rochester, MN

Metastatic breast cancer often results in debilitating osteolytic bone lesions that are the result of increased osteoclast activity. It has been observed that osteolysis is a localized phenomenon, occurring only in the area surrounding the tumor. This led us to believe that growth factors secreted by the tumor may be responsible for inducing differentiation of osteoclasts at the sites of osteolysis. To test this hypothesis, we have analyzed tumors from patients with significant osteolytic lesions for the presence of several growth factors, investigated the timing of the appearance of the factors at tumor sites using a mouse model, and examined the effects of the identified factors on osteoclastogenesis *in vitro*. Analysis revealed IGF-II, GM-CSF, TNF-α, and PTHrP protein and mRNA in the human tumors. We have examined the timing of growth factor appearance using a mouse model in which human MDA MB 231 cells are injected into the heart of athymic mice. mRNA was analyzed 1, 2, 3, and 4 weeks after injection. The incidence of PTHrP, human GM-CSF, human IGF II, and human TGF-ß mRNAs increased with tumor development, whereas TNF-α mRNA showed no such trend. Interestingly, mouse GM-CSF mRNA declined while human GM-CSF mRNA increased with each week. This pattern corresponds to increasing tumor size and suggests that the tumor may be repressing mouse GM-CSF production by a feedback inhibitory mechanism. To study effects of these growth factors on osteoclast differentiation, we used an *in vitro* model in which mouse osteoclast-like cells (mOCLs) are differentiated from precursors in the presence of growth factors. Treatment with IGF-I, PTHrP, or TNF-α stimulated and murine GM-CSF completely inhibited differentiation of mOCLs. Surprisingly, combined treatment with all four growth factors was also completely inhibitory. Taken together with the *in vivo* decline in mouse GM-CSF production seen at week 4, these results indicate that GM-CSF is a critical regulator of mOCL differentiation and that localized repression of GM-CSF at the sites of osteolytic lesions may be important in the observed localized increase in osteoclast numbers.

ABSTRACT: American Society for Bone and Mineral Research, 1999

TNF-α STIMULATES OSTEOCLAST MATURATION AND INHIBITS APOPTOSIS OF MATURE OSTEOCLASTS: A MODEL FOR TUMOR OSTEOLYSIS

A Shaw[1], A Gingery[2], J Maki[1], D A Pascoe[1], J Holy[3] and M J Oursler[1]
[1]Biology, [2]Biochemistry and Molecular Biology, and [3]Anatomy and Cell Biology; University of Minnesota, Duluth, MN.

Athymic mice inoculated with the human breast tumor cell line MDA MB 231 develop osteolytic lesions. We have previously demonstrated that osteolytic lesions are associated with an increase in osteoclast number, size, and activity. Additionally, we have shown that conditioned medium from the MDA MB 231 cell line is rich in many growth factors, including tumor necrosis alpha (TNF-α). These observations led us to propose that TNF-α may influence osteoclast differentiation or promote osteoclast survival by delaying apoptosis. To test this hypothesis, we have analyzed the effects of TNF-α on differentiation of mouse osteoclast-like cells (mOCLs) in vitro, measured the activity of TNF-α-induced mOCLs, and analyzed TNF-α-induced mOCLs for signs of apoptosis. Differentiation of mOCLs in the presence of TNF-α results in more mOCLs as well as mOCLs with more nuclei when compared to untreated mOCLs. Mature TNF-α-induced mOCLs were purified to remove mononuclear precursors and maintained in the absence of TNF-α for activity assays. Analysis of the conditioned medium from these larger mOCLs revealed an increase in cathepsin B, TRAP, and TGF-β secretion per cell as compared to untreated mOCLs. These larger cells were also shown to be more active in generating pits when laid onto bone. Mature mOCLs differentiated in the presence of TNF-α showed a decline in apoptosis markers within 30 minutes after removal of mononuclear precursors and continued TNF-α treatment, when compared to cells from which TNF-α was withdrawn. Taken together with previous findings that metastatic breast cancer increases the number, size, and activity of osteoclasts in vivo, these findings suggest a model for tumor osteolysis, in which TNF-α secreted by the tumor may be inducing differentiation as well as inhibiting apoptosis of osteoclasts.

ABSTRACT: American Society for Bone and Mineral Research, 2000

TNF-alpha Rapidly Induces Osteoclast Apoptosis by Direct Activation of Effectors and Suppression of Survival Pathways. Anne Gingery, Aubie Shaw, Jon Holy, and Merry Jo Oursler

Recently, much attention has been focused on the mechanisms by which osteoclasts regulate their survival. In order to better understand these mechanisms and how tumor necrosis factor (TNF)-alpha impacts osteoclast survival, we have employed in vitro generated mouse osteoclast-like cells as our model system. Osteoclasts generated with this system survived for several days once mature. When support cells were removed, the osteoclasts apoptosed within 24 to 48 hours. We have investigated the mechanism by which purified osteoclasts survive for many hours in the absence of support cells for this length of time. The addition of cycloheximide to the purified cultures caused a rapid 3-fold increase in apoptosis, indicating that protein synthesis is required for survival. We have examined second messenger phosphorylation to ascertain candidate signaling pathway involvement in survival regulation. Western blot analysis demonstrated phosphorylation of ERK1/2 and p38 MAP kinase within 15 minutes of purification. To further explore this, ERK 1/2 activation was blocked with PD 98059. This caused a 1.5-fold increase in apoptosis within 90 minutes of treatment. Blocking p38 MAP kinase with SB 20358 did not alter survival. Since PI3kinase activation promotes survival of many cells, we also examined the effects of chemical blocking of this pathway with wortmannin. Within 90 minutes of treatment, wortmannin caused a 1.5-fold increase in apoptosis levels. We conclude from these data that purified osteoclasts survive for a period following removal of support cells at least in part may be through activation of ERK 1/2 and PI3 kinase pathways.

We next examined the effects of TNF-alpha treatment on purified osteoclast survival. Addition of 0.05 ng/ml TNF-alpha caused apoptosis within 90 minutes. Blocking protein synthesis with cycloheximide did not prevent this effect, supporting that TNF-alpha induction of apoptosis is through direct activation of apoptosis effectors. Mature osteoclasts expressed TNF-alpha receptors RI and RII. Two lines of evidence support that RI is the receptor responsible for this response: (1) both human and mouse TNF-alpha caused this response and human TNF-alpha can only activate RI and (2) an antibody to the extracellular domain of RI mimicked the effects of TNF-alpha on apoptosis. We also studied TNF-alpha effects on osteoclast phosphorylation of ERK 1/2. TNF-alpha treatment resulted in significantly lower phosphorylation levels. We conclude from these data that TNF-alpha activates apoptosis through direct effects on apoptosis effectors via RI while acting to suppress survival mechanisms that are initiated following removal of support cells.

ABSTRACT: 2000 Department of Defense Breast Cancer Research Program ERA of HOPE meeting

Identification Of Breast Cancer Cell Line-Derived Paracrine Factors That Increase Osteolysis. A. Gingery, A. Shaw, L. Pederson, B. Winding, N. T. Foged, T. C. Spelsberg, and M. J. Oursler. 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 media secretes transforming growth factor type betas (TGF-ß1 and TGF-ß2), macrophage colony stimulating factor (M-CSF), granulocyte macrophage stimulating factor (GM-CSF), interleukins (IL-1 and IL-6), tumor necrosis factor alpha (TNF-α), insulin like growth factor II (IGF II), and parathyroid hormone related peptide (PTHrP). 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 media 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-ß2, and GM-CSF. No TGF-ß1 or IL-1 was detected in any of the active fractions. Our data supports the hypothesis that breast cancer cells modulate osteoclast activity using multiple regulatory factors that increased 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.

Bibliography

I. Publications:

Pederson, L. Winding, B., Foged, N.T., Spelsberg, T.C. and Oursler, M.J. (1999). Identification of breast cancer cell line-derived paracrine factors that stimulate osteoclast activity. Cancer Research 59:5840-5855.

Shaw, A. and Oursler, M.J. (2000). Paracrine regulation of osteoclast activity in metastatic breast cancer. Cancer Research Alert 1:124-127.

II Abstracts

American Society for Bone and Mineral Research 1998. Metastatic Breast Cancer: Indentification of Factors Involved in Tumor Progression in Bone. A. Shaw, M. Jakobsen, M. Rock, J. Ingle, B. Winding, M.J. Oursler.

American Society for Bone and Mineral Research 1999. TNF-α Stimulates Osteoclast Maturation and Inhibits Apoptosis of Mature Osteoclasts: a Model for Tumor Osteolysis. A. Shaw, A. Gingery, J. Maki, D.A. Pascoe, J. Holy, M.J. Oursler.

American Society for Bone and Mineral Research 2000. TNF-α Rapidly Induces Osteoclast Apoptosis by Direct Activation of Effectors and Suppression of Survival Pathways. A. Gingery, A. Shaw, J. Holy, M.J. Oursler.

Department of Defense Breast Cancer Research Program Era of Hope Meeting, 2000. Identification Of Breast Cancer Cell Line-Derived Paracrine Factors That Increase Osteolysis. A. Gingery, A. Shaw, L. Pederson, B. Winding, N. T. Foged, T. C. Spelsberg, and M. J. Oursler

III Personnel

Merry Jo Oursler, P.I.

David Pascoe, Scientist (M.S., Biology)

Aubie Shaw, Graduate Student

Mary Karst, Graduate Student

Anne Gingery, Graduate Student

Undergraduates employed as dishwashers in the laboratory (Laurie Yunker, Autumn Akkerman, Ronica Wick)

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the technical reports listed at enclosure. Request the limited distribution statement for these reports be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Reports to be Downgraded to Unlimited Distribution

ADB241560	ADB253628	ADB249654	ADB263448
ADB251657	ADB257757	ADB264967	ADB245021
ADB263525	ADB264736	ADB247697	ADB264544
ADB222448	ADB255427	ADB263453	ADB254454
ADB234468	ADB264757	ADB243646	
ADB249596	ADB232924	ADB263428	
ADB263270	ADB232927	ADB240500	
ADB231841	ADB245382	ADB253090	
ADB239007	ADB258158	ADB265236	
ADB263737	ADB264506	ADB264610	
ADB239263	ADB243027	ADB251613	
ADB251995	ADB233334	ADB237451	
ADB233106	ADB242926	ADB249671	
ADB262619	ADB262637	ADB262475	
ADB233111	ADB251649	ADB264579	
ADB240497	ADB264549	ADB244768	
ADB257618	ADB248354	ADB258553	
ADB240496	ADB258768	ADB244278	
ADB233747	ADB247842	ADB257305	
ADB240160	ADB264611	ADB245442	
ADB258646	ADB244931	ADB256780	
ADB264626	ADB263444	ADB264797	