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         Differentiation

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13. ABSTRACT (Maximum 200 Words)

Breast cancer fatally metastasizes to bone and activates osteoclasts, cells that resorb bone, resulting in the formation of osteolytic lesions. Certain drugs, bisphosphonates, slow the action of osteoclasts, however, the bone lesions are not repaired.

The osteoblasts should be able to repair the lesions by synthesizing new bone matrix. Instead, these cells appear to be inactivated by breast cancer, and the lesions do not heal. The purpose of this proposal was to understand what happens to osteoblasts in the presence of breast cancer. We hypothesized that breast cancer cells prevent pre-osteoblasts from completely maturing to osteoblasts. Our goals were to examine the effects of breast cancer cells on osteoblast proliferation, differentiation, and mature function. Using an osteoblast cell line and metastatic breast cancer cells, we found that conditioned medium from breast cancer cells inhibited osteoblast differentiation, as demonstrated by an inhibition of alkaline phosphatase, bone sialoprotein, and osteocalcin mRNA expression, and an inhibition of mineralization. These effects were mediated through TGFβ present in the conditioned medium. We also found that MDA-MB-231 conditioned medium altered osteoblast morphology, actin stress fiber formation, and presence of focal adhesion plaques. TGFβ, PDGF, and IGFII, all present in the conditioned medium, caused these effects by signaling through PI3K and rac.

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INTRODUCTION

Breast cancer frequently metastasizes to bone where it disrupts the delicate balance between osteoblasts (the bone forming cells) and osteoclasts (bone resorbing cells). Osteolytic lesions form at the site of invasion as a result of osteoclast activation. Certain drugs, bisphosphonates, are presently being used in an effort to block osteoclast function. These drugs slow lesion progression; however, the surrounding osteoblasts appear to be inactive and do not repair the lesions. We hypothesized that breast cancer cells alter the ability of osteoblasts to differentiate into mature, functional, matrix-producing cells. The aims of this proposal were 1) To determine if the growth phase of osteoblasts will be altered in the presence of breast cancer cells; 2) To determine if production of the osteoblast differentiation proteins alkaline phosphatase, bone sialoprotein and osteopontin are altered during the differentiation of osteoblasts in the presence of breast cancer cells or conditioned medium; 3) To determine if osteoblasts retain the ability to lay down a bone matrix and produce mature osteoblast proteins after exposure to breast cancer. To test this idea, conditioned medium from a bone-metastatic breast cancer cell line, MDA-MB-231, was cultured with osteoblasts that can differentiate in culture. These osteoblasts have been analyzed for their ability to proliferate, secrete matrix proteins, and form a mineralized matrix in the presence of MDA-MB-231 conditioned medium.

BODY

As described in the previous report, we chose to carry out these experiments using the murine osteoblast cell line, MC3T3-E1, instead of the human fetal osteoblast cell line, hFOB 1.19.

Task 1: To determine if the growth phase of osteoblasts will be altered in the presence of breast cancer cells. (Months 1-6)

   a. *Breast cancer cell conditioned media will be added to osteoblasts at the beginning, middle and end of the hFOB 1.19 growth cycle. Osteoblasts will be isolated, stained with crystal violet, and cell number determined. Growth will be measured at regular intervals on days 1,3,5 and 7. (month 1)*

   Details of this experiment were described in the previous report. MC3T3-E1 osteoblasts were cultured with conditioned medium from MDA-MB-231 cells. Cell number was assayed indirectly using the Promega Cell Titer assay. MDA-MB-231 conditioned medium caused an increase in cell number when compared to vehicle control treated cells. Additionally, MC3T3-E1 osteoblasts cultured with conditioned medium from NIH 3T3 fibroblasts did not affect cell number.

   b. *Task 1b-1c:* These aims were not pursued. Because we found strong effects culturing MC3T3-E1 osteoblasts with MDA-MB-231 conditioned medium, we chose instead to further identify the active factors in the conditioned medium, as well as the signaling pathway these factors utilized in the MC3T3-E1 osteoblasts. These experiments are described at the end of this report.

   c. *Determine if breast cancer cells or conditioned medium inhibits the growth of primary osteoblasts. (months 4-6)*

   It is difficult for our lab to obtain human primary osteoblasts. On two separate occasions, we received a flask of cells from a collaborator. MDA-MB-231

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conditioned medium also caused a modest, 2-fold increase in cell number, similar to that observed with MC3T3-E1 osteoblasts.

Task 2: To determine if the production of alkaline phosphatase, bone sialoprotein, and osteopontin are altered during the differentiation of osteoblasts in the presence of breast cancer cells or conditioned medium.
(Months 7-28)

     a. Detect alterations in osteoblast mRNA production of the differentiation proteins alkaline phosphatase, bone sialoprotein, and osteopontin caused by the addition of breast cancer cell conditioned media. Conditioned media will be added during the beginning, middle, and late stages of osteoblast differentiation, and analysis will be performed using Northern blot and RT-PCR. (months 7-9)

MC3T3-E1 osteoblasts were cultured with MDA-MB-231 conditioned medium and assayed for mRNA levels of alkaline phosphatase, osteocalcin, osteopontin and bone sialoprotein. mRNA was isolated every other day for up to 30 days of culture. Expression levels for both bone sialoprotein and osteocalcin were greatly downregulated in response to MDA-MB-231 conditioned medium. Although these results were obtained using 50% conditioned medium, the same effects were seen in a dose dependant manner with 5% to 35% conditioned medium.

We also examined alkaline phosphatase activity. The cells were cultured with 50% conditioned medium from either MDA-MB-231 cells or NIH 3T3 fibroblasts, or with 50% vehicle control medium. NIH 3T3 fibroblast conditioned medium was used as a negative control. MC3T3-E1 cells had no detectable alkaline phosphatase activity when cultured with MDA-MB-231 cell conditioned medium, but activity was not affected by NIH 3T3 fibroblast conditioned medium or vehicle control medium. Cells were examined at various times up to 35 days of culture.

Tasks 2b-2c: These experiments were not pursued for the reason described in Task 1b.

Tasks 2d-2g: Initially, we wanted to determine if breast cancer conditioned medium could alter either the protein expression or mRNA levels of these osteoblast differentiation proteins. Because we found a complete inhibition in mRNA levels, we have not pursued looking at the protein expression.

Task 2h: Using human primary osteoblasts, alkaline phosphatase activity was assayed as described for MC3T3-E1 osteoblasts. We observed a similar trend in that there was an inhibition of alkaline phosphatase activity in osteoblasts treated with MDA-MB-231 conditioned medium.

Task 3: To determine if osteoblasts retain the ability to lay down bone matrix and produce mature osteoblast proteins (Type I collagenase and osteocalcin) after exposure to breast cancer. (months 29-32)

     a. Add breast cancer cells or conditioned media to hFOBs as in task 2a and assay for bone nodule formation using Von Kossa staining of osteoblasts in co-culture, conditioned media, or a transwell system. (month 29)

MC3T3-E1 cells mineralize in culture, which can be visualized using the Von Kossa stain. When cultured with conditioned medium from MDA-MB-231 cells,

mineralization is greatly decreased compared to controls (vehicle control medium and NIH 3T3 fibroblast conditioned medium).

Task 3c: MDA-MB-231 conditioned medium inhibits osteocalcin expression, as described in Task 2a.

As described in the previous report, MDA-MB-231 conditioned medium altered MC3T3-E1 morphology, actin stress fiber formation (determined by phalloidin staining), and decreased the number of focal adhesion plaques (determined by interference reflection microscopy). To determine how MDA-MB-231 conditioned medium was causing these effects, MC3T3-E1 osteoblasts were pre-treated with $10\mu m$ LY294002, a PI3Kinase inhibitor, and then cultured with MDA-MB-231 conditioned medium. When treated with MDA-MB-231 conditioned medium, the osteoblasts became elongated and spindle shaped. However, osteoblasts treated with LY294002 and MDA-MB-231 conditioned medium had a cuboidal, cobblestone morphology similar to vehicle control treated cells. In addition, LY294002 reversed the alterations in actin stress fiber formation. When treated with MDA-MB-231 conditioned medium, the actin filaments in MC3T3-E1 osteoblasts were primarily cortical and punctate instead of being stretched into stress fibers. Osteoblasts treated with LY294002 and MDA-MB-231 conditioned medium had stress fiber formation similar to vehicle control treated cells. Finally, MDA-MB-231 conditioned medium also reduced the number of focal adhesion plaques in MC3T3-E1 osteoblasts, while pre-treatment with LY294002 reversed this effect. These results suggest that MDA-MB-231 conditioned medium alters osteoblasts through a PI3K pathway.

To identify the downstream target of PI3K, MC3T3-E1 osteoblasts were co-transfected with a GFP plasmid and a dominant negative plasmid to either of the small G-proteins, rac, rho or cdc42. These cells were fixed in paraformaldehyde and stained with phalloidin to visualize f-actin. Only those cells which received the GFP plasmid were examined. MC3T3-E1 osteoblasts transfected with dominant negative rho or cdc42 and treated with MDA-MB-231 conditioned medium had actin filaments similar to cells treated only with MDA-MB-231 conditioned medium. Osteoblasts transfected with dominant negative rac and treated with MDA-MB-231 condtioned medium had actin filaments similar to empty vector control cells. Thus, the data collectively show that MDA-MB-231 conditioned media affect MC3T3-E1 osteoblasts through PI3K and rac.

To determine the active factors present in MDA-MB-231 conditioned medium responsible for these effects, we used neutralizing antibodies to transforming growth factor β (TGFβ), platelet derived growth factor (PDGF), and insulin-like growth factor (IGFII), three cytokines known to alter actin filaments and focal adhesions through PI3K and rac. MDA-MB-231 conditioned medium was pre-incubated with each of these neutralizing antibodies for 1 hour at 37°. MC3T3-E1 osteoblasts were cultured with the neutralized conditioned medium and then assayed for stress fiber formation (phalloidin staining) and presence of focal adhesion plaques. Neutralizing each cytokine singly or in dual combinations had no effect on actin filaments or focal adhesion plaques. Only when all three cytokines were neutralized was there a restoration in actin stress fiber formation and the number of focal adhesion plaques.

To identify the factor in conditioned medium that inhibited osteoblast differentiation, MC3T3-E1 osteoblasts were cultured with MDA-MB-231 conditioned medium or vehicle control medium that was pre-incubated with a neutralizing antibody against TGFβ. In the presence of MDA-MB-231 conditioned medium and neutralizing TGFβ antibody, gene expression of bone sialoprotein, osteocalcin and alkaline phosphatase were all restored to control levels.

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Task 4: Data analysis and thesis preparation. These data were analyzed and compiled into a PhD thesis following the guidelines set forth by Pennsylvania State University. One paper was written and accepted in the peer reviewed journal Clinical and Experimental Metastasis. Another paper was written and submitted to Experimental Cell Research. These papers are both included in the Appendices.

Progress in Program: The final thesis defense was completed in fall, 2004. All revisions and paperwork were submitted to the graduate school in spring, 2005. All requirements for PhD have been completed. Graduation date was May 15, 2005.

Key Research Accomplishments:
- Discovered that breast cancer conditioned medium inhibits osteoblast differentiation
- The inhibition in differentiation is mediated through TGFβ
- Breast cancer conditioned medium altered stress fibers and reduced focal adhesion plaques
- These effects were mediated through the cytokines TGFβ, PDGF, and IGFII
- These cytokines signaled through PI3K and rac

Reportable Outcomes: (abstracts)

Mercer, R.R., Gay, C.V., Welch, D., Mastro, A.M. (2003) American Association for Cancer Research Annual Meeting "Identification of mechanisms involved in breast cancer induced apoptosis of osteoblasts" Proceedings of the 94th Annual Meeting of the American Association for Cancer Research, vol. 44:2412.

Mercer, R.R., Gay, C.V., Welch, D., Mastro, A.M. "Breast cancer cells downregulate alkaline phosphatase production in osteoblasts" Oncology (2003) 17, suppl 3 pg 54.

Mercer, R.R., Welch, D., Gay, C.V., Mastro, A.M. (2003) IVth International Conference on Cancer-Induced Bone Diseases "Breast cancer skeletal metastases affect osteoblast function.

Mercer, R.R., Chislock, E.M., Miyasaka, C., Welch, D., Gay, C.V., Mastro, A.M. (2004) American Association for Cancer Research Annual Meeting "Breast cancer skeletal metastases affect osteoblast function" vol. 45:5190.

Mercer, R.R., Welch, D., Gay, C.V., Mastro, A.M. AACR Pathobiology of Cancer workshop: *The Edward A. Smuckler Memorial Workshop* "Breast cancer skeletal metastases induces osteoblast apoptosis"

Publications:
Harms, J.F., Welch, D., Samant, R.S., Shevde, L.R., Miele, M.E., Babu, G.R., Melly, R., Beck, L.N., Kent, J., Gilman, V.R., Sosnowski, D.M., Campo, D.A., Gay, C.V., Budgeon, L.R., Christensen, N.D., **Mercer, R.R.**, Jewell, J., Mastro, A.M., Donahue, H.J., Erin, N., Paquette-Straub, C., Griggs, D.W., Kotyk, J.J., Pagel, M.D., Westlin, W.F., Rader, R.K. "A small molecule antagonist of the $\alpha_v\beta_3$ integrin suppresses MDA-MB-435 skeletal metastasis." Clinical and Experimental Metastasis (2004) in press.

Mastro, A.M., Gay, C.V., Welch, D., Donahue, H.J., Jewell, J., **Mercer, R.R.**, DiGirolamo, D., Chislock, E.M., Guttridge, K. (2004) "Breast Cancer Cells Induce Osteoblast Apoptosis: A Possible Contributor to Bone Degradation". J. Cell. Biochem. 91(2): 265-276.

Mercer, R.R., Miyasaka, C. and Mastro, A.M. (2004) "Bone Metastatic Breast Cancer Cells Suppress Osteoblast Adhesion and Differentiation" Clin. Exp. Mets. 21(5): 427-435.

Conclusions: Understanding how breast cancer cells affect osteoblasts following skeletal metastasis will be instrumental in finding new drug targets to not only treat osteolytic lesions, but to also prevent lesion formation. We found that MDA-MB-231 conditioned medium altered osteoblast morphology, stress fiber formation, reduced focal adhesion plaque formation. These effects were caused by TGFβ, PDGF, and IGFII, all present in MDA-MB-231 conditoined medium, and were mediated through PI3K and rac. In addition, TGFβ also prevented osteoblast differentiation, as seen by a substantial decrease in mRNA levels of alkaline phosphatase, bone sialoprotein, and osteocalcin, and a decrease in mineralization.

References: None

Appendix A
Mercer, R.R., Miyasaka, C. and Mastro, A.M. (2004) "Bone Metastatic Breast Cancer Cells Suppress Osteoblast Adhesion and Differentiation" Clin. Exp. Mets. 21(5): 427-435.

Appendix B
Robyn R. Mercer and Andrea M. Mastro "Cytokines secreted by bone-metastatic breast cancer cells alter the expression pattern of f-actin and reduce focal adhesion plaques in osteoblasts through PI3K" in submission (2005)

Metastatic Breast Cancer Cells Suppress Osteoblast Adhesion and Differentiation

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Breast Cancer Suppresses Osteoblast Function

ABSTRACT

Bone is a primary target for colonization of metastatic breast cancer cells. Once present, the breast cancer cells activate osteoclasts, thereby stimulating bone loss. Bone degradation is accompanied by pain and increased susceptibility to fractures. However, targeted inhibition of osteoclasts does not completely prevent lesion progression, nor does it heal the lesions. This suggests that breast cancer cells may also affect osteoblasts, cells that build bone. The focus of this study was to determine the ability of breast cancer cells to alter osteoblast function. MC3T3-E1 osteoblasts were cultured with conditioned medium from MDA-MB-231 breast cancer cells and subsequently assayed for changes in differentiation. Osteoblast differentiation was monitored by expression of osteocalcin, bone sialoprotein and alkaline phosphatase, and by mineralization. Osteoblasts cultured with MDA-MB-231 conditioned medium did not express these mature bone proteins, nor did they mineralize a matrix. Inhibition of osteoblast differentiation was found to be due to transforming growth factor β present in MDA-MB-231 conditioned medium. Interestingly, breast cancer conditioned medium also altered cell adhesion. When osteoblasts were assayed for adhesion properties using interference reflection microscopy and scanning acoustic microscopy, there was a reduction in focal adhesion plaques and sites of detachment were clearly visible. F-actin was disassembled and punctate in osteoblasts cultured with MDA-MB-231 conditioned medium rather than organized in long stress fibers. Taken together, these observations suggest that metastatic breast cancer cells alter osteoblast adhesion and prevent differentiation. These affects could account for the continued loss of bone after osteoclast inhibition in patients with bone-metastatic breast cancer.

Breast Cancer Suppresses Osteoblast Function

KEYWORDS: adhesion, bone, breast cancer, differentiation, metastasis, osteoblasts

ABBREVIATIONS: BSP bone sialoprotein; OCN osteocalcin; OSN osteonectin; IRM interference reflection microscopy; TGFβ transforming growth factor β

Breast Cancer Suppresses Osteoblast Function

Introduction

In the United States, the lifetime risk of a woman developing breast cancer is one in eight. Once the disease has progressed to its advanced stages, it metastasizes to bone 85% of the time and is usually fatal. Nonetheless, the process by which breast cancer affects bone tissue is poorly understood [1].

In normal bone tissue, the skeleton continuously undergoes remodeling; bone is resorbed by osteoclasts and deposited by osteoblasts in a balanced fashion. This tightly regulated balance between osteoclasts and osteoblasts is disrupted in bone diseases such as osteoporosis, rheumatoid arthritis, and osteolytic metastasis. When breast cancer metastasizes to bone, the osteoclasts are constitutively activated, resulting in osteolytic lesions [2]. These lesions cause many complications, including pain, bone fractures, and hypercalcemia [3]. Bisphosphonates can be used to block the activity of osteoclasts and help curb lesion progression, although the bone does not regain the ability to heal itself. Ordinarily, osteoblasts would produce new matrix to replace lost bone, but this does not happen [4]. One group examined nude mice with bone metastases and found a significant *decrease* in bone formation [5]. Other researchers have reported similar results [4, 6, 7]. Therefore, it appears likely that breast cancer cells affect osteoblasts in addition to osteoclasts.

Breast cancer cells can limit osteoblasts by either 1) inducing osteoblast apoptosis, and/or 2) interfering with normal osteoblast function. Using an *in vitro* system, we recently reported that bone metastatic breast cancer cells increased the

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percentage of osteoblasts undergoing apoptosis [8]. Fromigue et al. also reported an increase in apoptosis in bone marrow stromal cells exposed to breast cancer cell conditioned medium [9]. Although many apoptotic mechanisms were explored, we have not yet determined the specific mechanism used by the breast cancer cells to induce osteoblast apoptosis.

There are a limited number of studies that suggest that breast cancer cells may affect osteoblasts in various ways. One group examined osteoblast proliferation in the presence of breast cancer cell conditioned medium [10, 11]. They found that the conditioned medium inhibited osteoblast proliferation and increased their sensitivity to osteolytic agents, such as parathyroid hormone. Two reports found that MCF7 cells, a poorly metastatic breast cancer cell line, inhibit osteocalcin mRNA expression in osteoblasts [12, 13]. However, a more comprehensive analysis of osteoblast differentiation in response to bone metastatic breast cancer cells has not been carried out.

In this present study, we examined the ability of metastatic breast cancer cells to affect osteoblast function. An osteoblast cell line, MC3T3-E1, was cultured with conditioned medium from the human metastatic breast cancer cell line, MDA-MB-231. MC3T3-E1 cells are an immature osteoblast cell line isolated from mouse calvaria that can differentiate and mineralize in culture [14]. MDA-MB-231 cells are a breast cancer line [15] that can metastasize to bone and cause osteolytic lesions following injection into the left cardiac ventricle [16]. Using a battery of approaches, we found that in response to breast cancer conditioned medium, osteoblasts exhibited altered adhesion properties as well as a loss of the ability to differentiate. These findings suggest that metastatic breast

cancer cells contribute to bone loss by altering osteoblast function, in addition to

stimulating osteoclast activity.

Breast Cancer Suppresses Osteoblast Function

Materials and Methods

Materials

Culture media, DMEM and α-MEM with glutamine and nucleosides, and penicillin/streptomycin were obtained from MediaTech (Herndon, VA). Monoclonal anti-β-tubulin cy3 conjugated antibody, Fast Blue RR salt and Napthol AS-BI phosphate were obtained from Sigma (St. Louis, MO). Neutralizing TGFβ antibody was purchased from R&D Systems (Minneapolis, MN). RNeasy mini kit was purchased from Qiagen (Maryland). AlexaFluor 568 phalloidin was obtained from Molecular Probes (Eugene, OR). Paraformaldehyde was purchased from Electron Microscopy Sciences (Ft. Washington, PA). All PCR primers were purchased from Integrated DNA technologies (Coralville, IA). QuantumRNA™ 18s Internal Standards were purchased from Ambion (Austin, TX).

Cells and culture conditions

MC3T3-E1 osteoblasts were a gift from Dr. Norman Karin, University of Delaware. MDA-MB-231 cells were a gift from Dr. Danny Welch, University of Alabama at Birmingham. The NIH 3T3 fibroblast cell line was obtained from the ATCC. Human primary osteoblasts were a gift from Dr. Henry Donahue, Penn State Hershey Medical Center. MDA-MB-231 cells were maintained in DMEM containing 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). MC3T3-E1 osteoblasts were maintained in α–MEM with glutamine and nucleosides plus 10% FBS and 1% P/S. All

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cells were cultured in a humidified 37° incubator containing 5% CO_2 and 95% air. To collect conditioned medium from MDA-MB-231 cells, the cells were plated in T-175 flasks and cultured to ~90% confluence. The media in each flask was then replaced with 20ml of serum free α-MEM. After 24 hours, the conditioned medium was removed, centrifuged at 1000 rpm for 10 minutes, pooled, aliquoted, and frozen at $-20^\circ C$ until use.

For all experiments, MC3T3-E1 osteoblasts were plated at 10^4 cells/cm^2 in differentiation medium (α-MEM + 10% FBS, 50ug/ml ascorbic acid, and 10mM β-glycerophosphate) and incubated overnight. The following day, media were replaced with 50% 2x differentiation medium (α-MEM + 20% FBS, 100ug/ml ascorbic acid, and 20mM β-glycerophosphate) plus 50% MDA-MB-231 conditioned medium or vehicle control medium (serum free medium). Cells were cultured the indicated number of days, receiving media changes every other day.

Cytoskeletal staining

MC3T3-E1 osteoblasts were cultured with MDA-MB-231 conditioned medium or vehicle control medium for 7 days. Cells were fixed in 4% paraformaldehyde and stained with either AlexaFluor 568 phalloidin (20 minutes) or monoclonal anti-β-tubulin cy-3 conjugate (1 hour). Cells were visualized with a confocal microscope.

Interference reflection microscopy

Breast Cancer Suppresses Osteoblast Function

A Bio-Rad MRC1024 confocal microscope supported by a Dell Power Edge 1600SC with a blue reflection filter was used to generate interference reflection images. MC3T3-E1 osteoblasts were cultured with 50% conditioned medium from MDA-MB-231 cells or 50% vehicle control medium on glass coverslips. Cells were imaged live following 3 days of culture.

Scanning acoustic microscopy

MC3T3-E1 osteoblasts were cultured with 50% conditioned medium on plastic coverslips. Acoustic images were generated with a UH3 scanning acoustic microscope operated at 400 MHz frequency. The acoustic lens was made of sapphire with a diameter curvature of 500um and an opening half-angle of 60°. The acoustic transducer lens was made of zinc oxide.

RNA isolation and RT-PCR

MC3T3-E1 osteoblasts were cultured up to 30 days with MDA-MB-231 conditioned medium or vehicle control medium. RNA was isolated every other day using the RNeasy kit and analyzed using RT-PCR. RNA (100ng) was added to the reverse transcription reaction. Primers used: osteonectin 5'CTGCCTGCCTGTGCCGAGAGTTCC3' (sense) and 5'CCAGCCTCCAGGCGCTTCTCATTC3' (anti-sense); osteocalcin 5'CAAGTCCCACACAGCAGCTT3' (sense) and 5'AAAGCCGAGCTGCCAGAGTT3' (anti-sense); bone sialoprotein 5'AACAATCCGTGCCACTCA3' (sense) and

5'GGAGGGGGCTTCACTGAT3' (anti-sense). As a loading control, either actin or

QuantumRNA™ 18s Internal Standards were co-amplified with the genes.

Alkaline phosphatase activity

MC3T3-E1 osteoblasts were cultured 35 days with 50% MDA-MB-231 conditioned

medium or vehicle control medium. Cells were fixed in 10% formaldehyde and stained

using Fast Blue RR Salt and Napthol AS-BI Phosphate for 30 minutes at 37°C. Cells

were rinsed in water and mounted for microscopy.

Mineralization

MC3T3-E1 osteoblasts were cultured as described for alkaline phosphatase activity. Von

Kossa staining was done by first fixing the cells in 10% formaldehyde (in phosphate

buffered saline) and then staining in 5% silver nitrate for 30 minutes in the dark. Cells

were washed and mounted for microscopy.

ELISA

TGFβ was quantitated using a sandwich ELISA. Flat-bottom 96-well plates were coated

with TGFβ antibody (R&D Systems MAB1835) at 2μg/ml and incubated overnight at

4°C. The plates were washed four times with PBS with 0.05% Tween 20 and blocked for

2 hours with PBS and 1% BSA. After washing three times, samples and standards were

added and incubated overnight at 4°C. The plates were then washed four times and

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incubated with TGFβ detection antibody (TGFβ$_1$: R&D Systems BAF240; TGFβ$_2$: R&D Systems BAF302) at 50μg/ml for 2 hours at room temperature. The plates were washed 6 times and incubated with NeutrAvidin horseradish peroxidase conjugate (Pierce #31001) for 30 minutes at room temperature. The plates were washed 8 times and then incubated with ABTS peroxidase substrate at room temperature for 90 minutes. Plates were read at 405nm in an ELISA reader.

Results

Cell morphology and adhesion

MC3T3-E1 osteoblasts cultured to confluence were cuboidal and the culture displayed a patterned, cobblestone appearance (Figure 1). This pattern changed when the osteoblasts were cultured with conditioned medium from MDA-MB-231 cells. Under these conditions, the cells became long, spindle-like, and assumed a fibroblastic morphology. To further examine the change in shape, the cytoskeleton was analyzed by staining actin stress fibers (Figure 2) and β-tubulin formation (Figure 3). MC3T3-E1 osteoblasts displayed strong stress fiber formation, as indicated by f-actin staining. However, the cells cultured with MDA-MB-231 conditioned medium showed few stress fibers; rather, the staining was primarily cortical and punctate. Staining for β-tubulin revealed no differences between MC3T3-E1 osteoblasts cultured with MDA-MB-231 conditioned medium and those cultured with vehicle control medium.

A disruption in stress fiber formation suggested that the osteoblasts might have difficulty forming focal adhesion plaques. Focal adhesion plaques (areas of concentrated adhesion proteins) will reflect light and thus can be visualized using interference reflection microscopy. By this procedure, focal adhesion plaques appeared as black spots at sites of adhesion, primarily focused around the outer edge of the cell. MC3T3-E1 osteoblasts were assayed for adhesion plaques following culture with MDA-MB-231 conditioned medium. All of the MC3T3-E1 osteoblasts cultured in vehicle control medium had many

strong focal adhesion plaques (Figure 4). In contrast, only a few MC3T3-E1 osteoblasts

cultured with conditioned medium from MDA-MB-231 cells had focal adhesion plaques.

Moreover, those cells that did have focal adhesion plaques had a small number per cell.

To further characterize adhesion, the cells were examined by scanning acoustic

microscopy. A scanning acoustic microscope emits sound waves that vibrate through

both the cells and their culture medium and across the surface of the plastic substrate.

The image received indicates areas of cell attachment to the substrate (dark gray to

black). The areas where the cells were not attached appeared white [17]. When the cells

were cultured with vehicle control medium, a patterned appearance of adhesion was

noted (Figure 5). However, cells cultured with MDA-MB-231 conditioned medium

displayed many regions of detachment, as indicated by the white patches.

Taken together, a lack of focal adhesion plaques, altered stress fiber formation, and sites

of detachment indicated that MDA-MB-231 conditioned medium altered MC3T3-E1

adhesion, likely responsible for their gross morphology.

Differentiation

Osteoblasts undergo a patterned differentiation process, beginning with the secretion of a

variety of non-collagenous matrix proteins and ending with mineralization. According to

Lian and Stein [18], the bone is normally covered with preosteoblast lining cells, which

differentiate into mature osteoblasts. Three phases of differentiation can be detected, including rapid proliferation, matrix maturation, and mineralization. Matrix maturation can be detected by a decrease in cell proliferation and by the secretion of alkaline phosphatase, bone sialoprotein, and osteopontin. The mineralization phase is marked by osteocalcin expression and nodule formation. Because bone formation by osteoblasts is tightly coupled with bone resorption by osteoclasts, the differentiation process is ongoing.

We analyzed MC3T3-E1 expression of a selected group of osteoblast proteins, including bone sialoprotein, osteocalcin, osteonectin, and alkaline phosphatase activity in the presence of MDA-MB-231 conditioned medium. In preliminary studies, we established the pattern of expression of these proteins in the MC3T3-E1 cells in our laboratory. Using RT-PCR, we found that bone sialoprotein expression was first observed between 7 and 9 days of culture, while osteocalcin expression was not detected until about 13 days. Osteonectin mRNA was detected in immature osteoblasts (3 days) and levels began to increase after 15 days (data not shown).

Therefore, MC3T3-E1 osteoblasts were cultured with MDA-MB-231 conditioned medium and assayed for mRNA levels of these proteins. mRNA was isolated every other day for up to 30 days of culture. Expression levels for both bone sialoprotein and osteocalcin were greatly downregulated in response to MDA-MB-231 conditioned medium (Figure 6, shown after 15 and 23 days of culture). Expression levels of mRNA for osteonectin were initially equivalent to cells cultured in vehicle control medium.

However, after 15 days of culture, mRNA levels in control cells were enhanced, but this did not occur in cells cultured with the cancer conditioned medium. Instead, osteonectin mRNA expression remained near the basal level in these cells, even after 30 days. Although these results were obtained using 50% conditioned medium, the same effects were seen in a dose dependant manner with 5% to 35% conditioned medium (data not shown).

We also examined alkaline phosphatase activity. In response to MDA-MB-231 conditioned medium, MC3T3-E1 osteoblasts had no detectable alkaline phosphatase activity when examined at various times up to 35 days of culture (Figure 7A and B).

The final step in determining the effects of MDA-MB-231 conditioned medium on MC3T3-E1 differentiation was analysis of their ability to mineralize a matrix. MC3T3-E1 osteoblasts were cultured for 35 days with MDA-MB-231 conditioned medium and then assayed by Von Kossa staining (Figure 7D and E). Mineralization was observed in cells cultured with vehicle control medium (large, black areas of phosphate crystals); however, mineralization appeared to be completely inhibited in cells cultured with MDA-MB-231 conditioned medium.

To determine the factors present in MDA-MB-231 conditioned medium responsible for the inhibition of osteoblast differentiation, we referred to the report of Pederson et al. [19]. They assayed MDA-MB-231 conditioned medium for the presence of a variety of cytokines and growth factors. Interestingly, they reported that MDA-MB-231

conditioned medium contained both transforming growth factor (TGF) β_1 and β_2, two

cytokines reported previously to inhibit osteoblast differentiation [20, 21]. Using ELISA,

we found that conditioned medium from MDA-MB-231 cells contained 5ng/ml of TGFβ_1

and 2ng/ml of TGFβ_2. To test for the role of TGFβ in the conditioned medium, we

neutralized TGFβ by pre-incubating the conditioned medium with a neutralizing

antibody. MC3T3-E1 osteoblasts were then cultured for 10 days prior to the addition of

MDA-MB-231 conditioned medium or conditioned medium treated with neutralizing

antibody to TGFβ. After an additional 6 days of culture with the conditioned media,

mRNA was isolated and analyzed for expression of alkaline phosphatase, bone

sialoprotein, and osteocalcin (Figure 8). Neutralization of TGFβ from MDA-MB-231

conditioned medium completely restored mRNA expression of these three genes.

In order to test for specificity, MC3T3-E1 osteoblasts were also cultured with

conditioned medium from 3T3 fibroblasts and assayed for changes in f-actin, alkaline

phosphatase activity, and mineralization. MC3T3-E1 osteoblasts cultured with 3T3

fibroblast conditioned medium appeared to be identical to those cells cultured with

vehicle control medium (Figure 7C and F, and data not shown). In addition, we cultured

human primary osteoblasts with conditioned medium from MDA-MB-231 cells. These

cells were subsequently assayed for alterations in f-actin and alkaline phosphatase

activity. Human primary osteoblasts cultured with MDA-MB-231 conditioned medium

displayed punctate f-actin staining and no detectable alkaline phosphatase activity, while

cells cultured with vehicle control medium had stress fiber formation and strong alkaline

phosphatase activity (Figure 9 and data not shown).

Discussion

In this study, we examined the effects of MDA-MB-231 conditioned medium on MC3T3-E1 osteoblasts. We found: 1) MDA-MB-231 conditioned medium altered MC3T3-E1 cell adhesion, as indicated by a lack of focal adhesion plaques and altered stress fiber formation; and 2) MDA-MB-231 conditioned medium blocked MC3T3-E1 differentiation, as determined by a decrease of bone sialoprotein, osteocalcin, and osteonectin mRNA expression, no alkaline phosphatase activity, and little mineralization. This study presents a novel approach to analyzing breast cancer metastasis. While research traditionally has focused on understanding how breast cancer cells stimulate osteoclast activity, this study clearly reveals that breast cancer cells also affect osteoblasts. The lack of osteoblast differentiation can lead to a decrease in bone formation, accelerating the bone loss associated with osteoclasts.

Culture of MC3T3-E1 osteoblasts with MDA-MB-231 conditioned medium completely disrupted the patterned differentiation process. The osteoblasts were unable to regulate expression levels of important matrix proteins, including bone sialoprotein, osteocalcin and osteonectin. Downregulation of these proteins *in vivo* would prevent osteoblasts from generating a mature matrix. In addition, alkaline phosphatase activity and mineralization, two markers of mature osteoblasts, were completely inhibited in MC3T3-E1 osteoblasts cultured with MDA-MB-231 conditioned medium. These data suggest that metastatic breast cancer cells secrete molecules that completely block the normal differentiation process of osteoblasts, preventing bone formation.

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Breast Cancer Suppresses Osteoblast Function

While others have examined the effects of breast cancer on osteoblasts, this study is the first to demonstrate that bone-metastatic breast cancer cells inhibit osteoblast differentiation from early protein expression through the mineralization step. In addition, cell morphology and adhesion are greatly altered. It was previously reported that breast cancer enhanced osteoblast differentiation; however, this study used a non-metastatic breast cancer cell line, HT-39 [12]. This group further found that conditioned medium from a bone-metastatic breast cancer cell line inhibited osteocalcin expression [12, 13]. Our findings support these latter results.

A variety of factors are in breast cancer conditioned medium, including TGFβ [19], a reported inhibitor of osteoblast differentiation. Specifically, it can inhibit production of alkaline phosphatase and osteocalcin [20, 21]. We demonstrated in this study that TGFβ present in MDA-MB-231 conditioned medium is at least in part responsible for the inhibition of osteoblast differentiation. To determine this, either MDA-MB-231 conditioned medium or conditioned medium with neutralized TGFβ was added to maturing osteoblasts (10 days old). The cells were then cultured for an additional 6 days (16 days total) and mRNA was isolated for RT-PCR. MC3T3-E1 osteoblasts cultured with the conditioned medium with neutralized TGFβ had restored mRNA expression of alkaline phosphatase, bone sialoprotein and osteocalcin. Because the MC3T3-E1 osteoblasts were allowed to culture 10 days prior to the addition of MDA-MB-231 conditioned medium, this experiment bypassed the alterations in cell adhesion that conditioned medium induces in early, immature osteoblasts. Therefore, it is possible that the alterations in cell adhesion could also in part be responsible for the inhibition in osteoblast differentiation in combination with TGFβ.

It remains to be determined how MDA-MB-231 conditioned medium completely disrupts osteoblast adhesion. Osteoblasts require proper adhesion and subsequently integrin signaling for differentiation to occur [22, 23]. The α_2 integrin can activate the transcription factor cbfa1, leading to osteoblast differentiation [24]. For osteoblast differentiation to occur, the cell requires an appropriate extracellular matrix and proper cell adhesion. We demonstrated that MC3T3-E1 osteoblasts cultured with MDA-MB-231 conditioned medium did not secrete a normal extracellular matrix and that cell adhesion was negatively affected. Osteoblasts cultured with MDA-MB-231 conditioned medium had difficulty forming stress fibers and showed a great reduction in the numbers of focal adhesion plaques. Therefore, we believe that MDA-MB-231 conditioned medium disrupted osteoblast adhesion. While we know that TGFβ is responsible for inhibiting differentiation in maturing osteoblasts, we found that it is not solely responsible for the effects seen in cell adhesion (data not shown). Therefore, how MDA-MB-231 conditioned medium completely inhibits osteoblast adhesion and differentiation remains a complex and multi-faceted process.

Because MC3T3-E1 osteoblasts are a cell line derived from a mouse, we were concerned that the data gathered may be species specific. We tested human primary osteoblasts and found that in response to the conditioned medium, these cells also displayed a morphology similar to that of MC3T3-E1 osteoblasts cultured with MDA-MB-231 conditioned medium. The primary cells were assayed for f-actin in response to either vehicle control medium or MDA-MB-231 conditioned medium, and the same trends were observed; the cells displayed only cortical, punctate staining when cultured with MDA-MB-231 conditioned medium (data not shown). We plan to continue

experiments with human primary osteoblasts to further validate the use of MC3T3-E1 osteoblasts.

To date, research has focused on finding ways to inhibit osteoclast activity in an effort to curb bone loss. While this is an important approach, the information obtained from our study indicates that the osteoblast is also affected by breast cancer tumors. A decrease in bone formation tethered with an increase in bone degradation will exacerbate bone loss associated with breast cancer metastasis. Focusing solely on osteoclast activity will not cure bone loss. Exploring the possibility of stimulating osteoblast activity is also needed and must be considered as an important step for therapeutic intervention.

Breast Cancer Suppresses Osteoblast Function

Acknowledgements

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Figure legends:

Figure 1. Morphology of MC3T3-E1 osteoblasts in the presence of MDA-MB-231 conditioned medium. MC3T3-E1 cells were cultured with 50% MDA-MB-231 conditioned medium for 3 and 5 days. Note the patterned cobblestone appearance of the vehicle control cells compared to the long, spindle-like morphology of cells cultured with the cancer cell conditioned medium.

Figure 2. F-actin in MC3T3-E1 osteoblasts cultured with MDA-MB-231 conditioned medium. MC3T3-E1 cells were cultured with 50% MDA-MB-231 conditioned medium for 3 days. F-actin was visualized using phalloidin; nuclei were stained with SYBR Green. A-C: vehicle medium treated cells; D-F: Cells treated with MDA-MB-231 conditioned medium. A and D: SYBR GR stain of nuclei; B and E: phalloidin stain of f-actin; C and F: overlay.

Figure 3. MC3T3-E1 microtubule formation in the presence of MDA-MB-231 conditioned medium. MC3T3-E1 osteoblasts were cultured with 50% MDA-MB-231 conditioned medium for 3 days and then stained with anti-β-tubulin to visualize microtubules and SYBR GR to visualize nuclei. A-C: vehicle control cells; D-F: Cells treated with MDA-MB-231 conditioned medium. A and D: SYBR GR stain of nuclei; B and E: anti-β-tubulin; C and F: overlay.

Figure 4. Focal adhesion plaques in MC3T3-E1 osteoblasts. MC3T3-E1 osteoblasts were cultured with 50% MDA-MB-231 conditioned medium or vehicle control medium for 48 hours. Focal adhesion plaques were visualized using interference reflection microscopy (IRM) and appear as black spots.

Figure 5. Attachment of MC3T3-E1 osteoblasts cultured with MDA-MB-231 conditioned medium. MC3T3-E1 osteoblasts were cultured with 50% vehicle control medium or 50% MDA-MB-231 conditioned medium for 48 hours. The cells were analyzed using a scanning acoustic microscope to indicate sites of attachment. Arrows indicate regions of detachment.

Figure 6. Expression of non-collagenous matrix proteins in MC3T3-E1 osteoblasts. MC3T3-E1 osteoblasts were cultured with 50% vehicle control medium or 50% MDA-MB-231 conditioned medium for up to 30 days. RNA was isolated and analyzed using RT-PCR for bone sialoprotein (bsp), osteocalcin (ocn), and osteonectin (osn). Lane 1: vehicle control medium day 15; lane 2: MDA-MB-231 conditioned medium day 15; lane 3: vehicle control medium day 23; lane 4: MDA-MB-231 conditioned medium day 23.

Figure 7. MC3T3-E1 differentiation following culture with MDA-MB-231 conditioned medium. MC3T3-E1 osteoblasts were cultured with 50% MDA-MB-231 conditioned medium or 50% 3T3 fibroblast conditioned medium for 35 days. Cells were fixed and assayed for alkaline phosphatase activity or for mineralization using Von Kossa. A) Alkaline phosphatase activity of cells cultured with vehicle control medium; B) Alkaline

phosphatase activity of cells cultured with MDA-MB-231 conditioned medium; C) Alkaline phosphatase activity of cells cultured with 3T3 fibroblast conditioned medium; D) Von Kossa staining of cells cultured with vehicle control medium; E) Von Kossa staining of cells cultured with 50% MDA-MB-231 conditioned medium; F) Von Kossa staining of cells cultured with 50% 3T3 fibroblast conditioned medium.
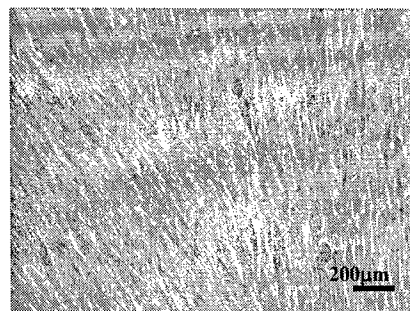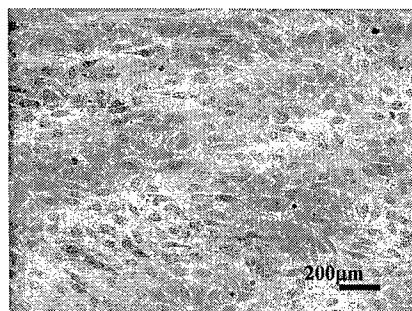
Figure 8. MC3T3-E1 mRNA expression following treatment with a neutralizing TGFβ antibody. MC3T3-E1 osteoblasts were cultured with 25% MDA-MB-231 conditioned medium or conditioned medium pre-incubated with a TGFβ neutralizing antibody. After 16 days of culture, mRNA was isolated and RT-PCR performed for alkaline phosphatase, bone sialoprotein and osteocalcin. Lane 1: vehicle control medium; Lane 2: vehicle control medium + anti-TGFβ; Lane 3: MDA-MB-231 conditioned medium; Lane 4: MDA-MB-231 conditioned medium + anti-TGFβ

Figure 9. Alkaline phosphatase activity in human primary osteoblasts following culture with MDA-MB-231 conditioned medium. Human primary osteoblasts were cultured with either 50% vehicle control medium or 50% MDA-MB-231 conditioned medium for 25 days. Cells were fixed and assayed for alkaline phosphatase activity. A) Vehicle control treated cells; B) MDA-MB-231 conditioned medium treated cells.
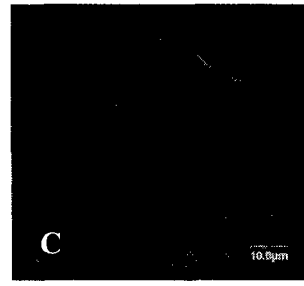
Day 3

Day 5

200μm 200μm

200μm 200μm

VM +231 CM

A   10.0μm   B   10.0μm   C   10.0μm

D   10.0μm   E   10.0μm   F   10.0μm
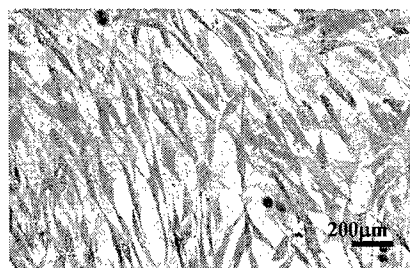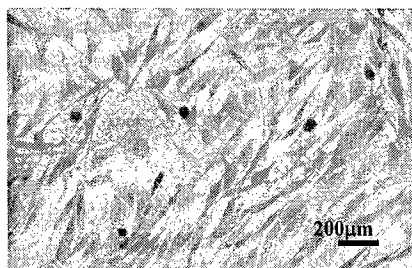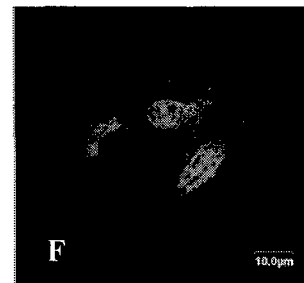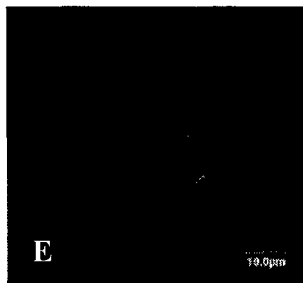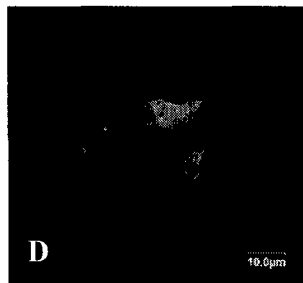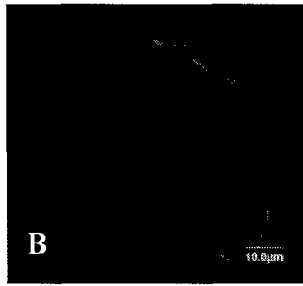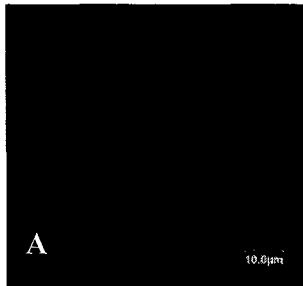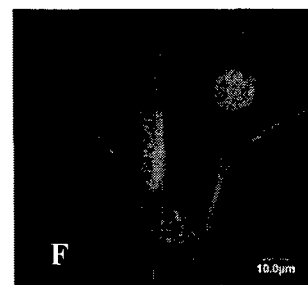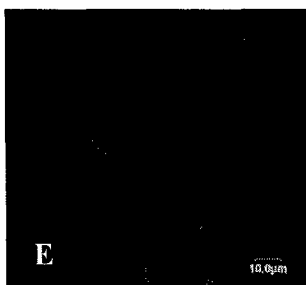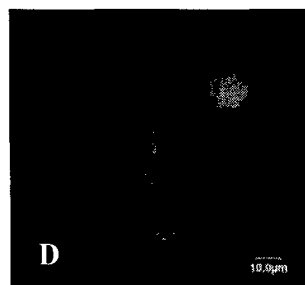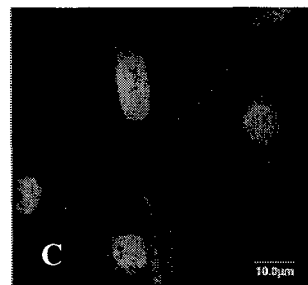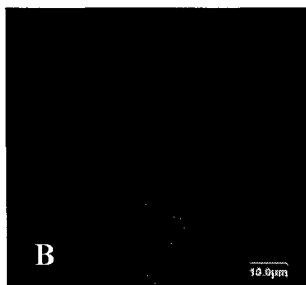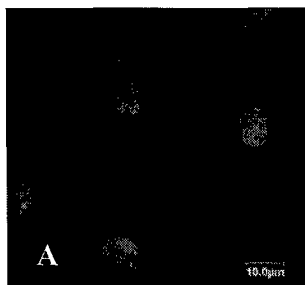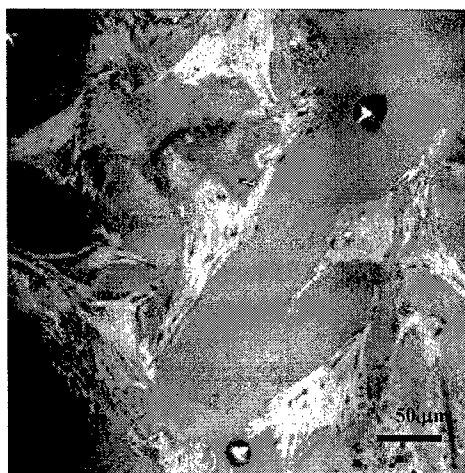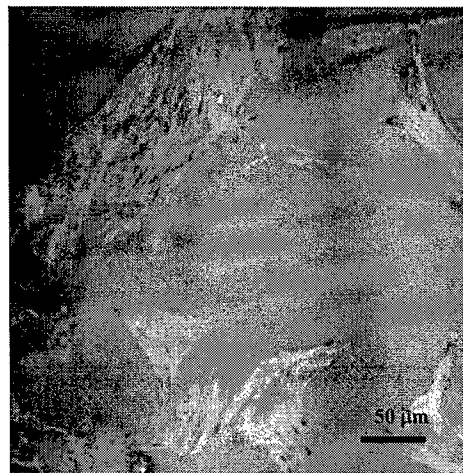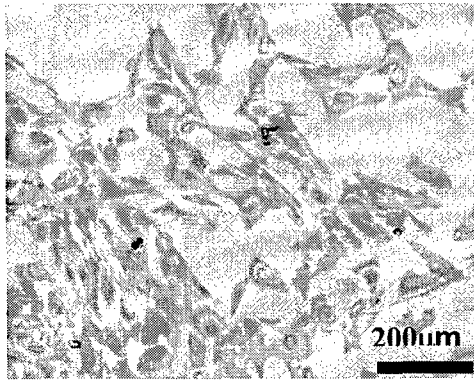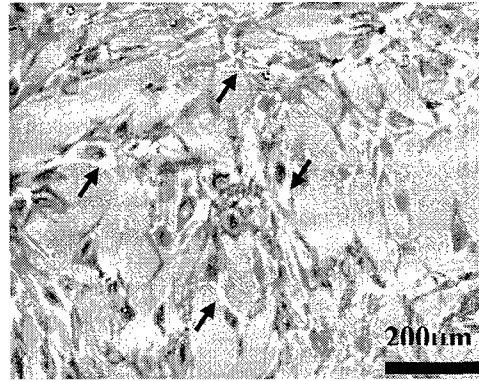
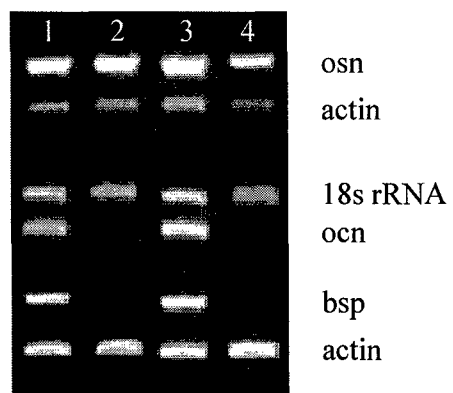Vehicle control   +MDA-MB-231
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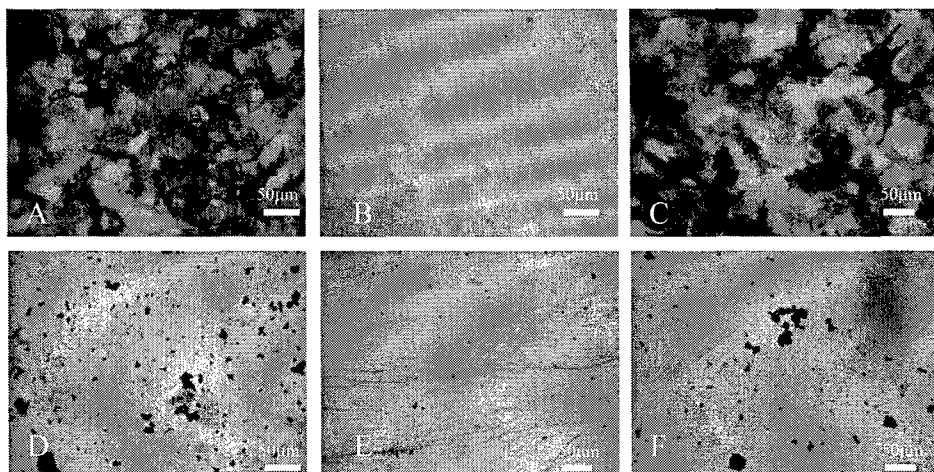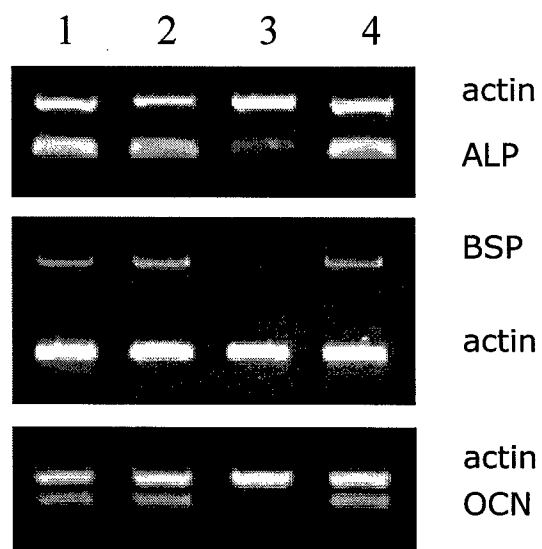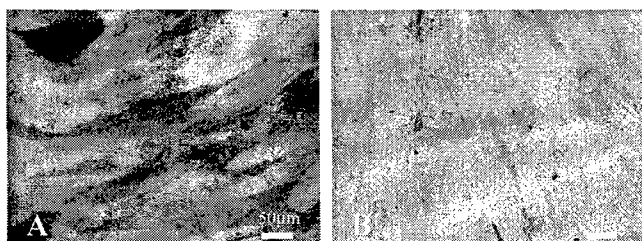
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actin

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Cytokines secreted by bone-metastatic breast cancer cells alter the expression pattern of f-actin and reduce focal adhesion plaques in osteoblasts through PI3K

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Running Head: Breast Cancer Alters Osteoblast Adhesion

ABSTRACT

Breast cancer frequently metastasizes to bone, resulting in osteolytic lesions. These lesions, formed by activated osteoclasts, cause pain, an increased susceptibility to fractures, and hypercalcemia. It has been shown that breast cancer cells communicate with osteoblasts and subsequently stimulate osteoclast activity; however, little research has focused on understanding the interaction between breast cancer cells and osteoblasts. We recently reported that conditioned medium from MDA-MB-231 breast cancer cells inhibited the differentiation of MC3T3-E1 osteoblasts through the secretion of transforming growth factor β (TGFβ). In addition, the breast cancer conditioned medium altered MC3T3-E1 morphology, the pattern of actin stress fibers, and reduced focal adhesion plaques. In the current study, we identified the mechanism used by MDA-MB-231 cells to cause these effects. When MC3T3-E1 osteoblasts were cultured with MDA-MB-231 conditioned medium preincubated with neutralizing antibodies to platelet derived growth factor (PDGF), insulin-like growth factorII (IGFII), and TGFβ, focal adhesion plaques and actin stress fiber formation were restored. These cytokines were further found to signal through PI3Kinase and Rac. In conclusion, TGFβ, PDGF, and IGFII might be good therapeutic targets for treating breast cancer-induced osteolytic lesions.

Keywords: osteoblast, breast cancer, cell adhesion, platelet derived growth factor, insulin-like growth factor, transforming growth factor β

INTRODUCTION

A woman in the United States has a lifetime risk of 1 in 8 of developing breast cancer [1]. In the year 2003, there was an estimated 215,990 new cases of invasive breast cancer, the most frequent non-skin cancer in women. The survival rate for a low grade, low stage tumor with no metastases to lymph nodes or other organs is greater than 95%. Patients with metastases to distant organs, including bone, have a 5-year survival rate of less than 20% [2].

When breast cancer metastasizes to bone, osteolytic lesions form [3]. These lesions are the result of activated osteoclasts, the cells that resorb bone. Patients with severe osteolytic lesions suffer from an increased susceptibility to fractures, unexplained bone pain, and hypercalcemia from the excess release of calcium stored in the bone matrix [4]. The current treatment for skeletal lesions is the administration of bisphosphonates to inhibit osteoclast activity. This strategy has been successful at slowing lesion progression; however, the lesions do not heal [5].

The fact that bone does not regenerate following bisphosphonate treatment suggests that breast cancer cells alter osteoblast function in addition to stimulating osteoclast activity. In a study of seven patients with humoral hypercalcemia of malignancy, bone biopsies were analyzed for both osteoclast and osteoblast activity. There was both evidence of increased osteoclast activity and reduced osteoblastic activity, as indicated by decreased osteoblastic surface, osteoid surface, and osteoid volume [6]. Other studies have reported similar results [7, 8]. Therefore, it is likely that breast cancer cells stimulate osteoclasts, but negatively affect osteoblasts.

Evidence obtained *in vitro* indicates that breast cancer cells can induce osteoblast apoptosis. Two human bone-metastatic breast cancer cell lines, MDA-MB-231 and MDA-MB-435, caused osteoblasts to undergo apoptosis through both direct cell-cell contact and through conditioned medium [9]. Fromigue et al. also reported that breast cancer cells induce apoptosis in bone marrow stromal cells [10].

Although breast cancer cells induce osteoblast apoptosis, the apoptotic rate is less than 10% of the total population. We recently found from an *in vitro* study that breast cancer cells inhibit osteoblast function of the remaining cell population [11]. When conditioned medium from MDA-MB-231 cells was cultured with MC3T3-E1 cells, an immature osteoblast cell line that can be induced to differentiate in culture, it prevented their differentiation, as noted by inhibition of the expression of alkaline phosphatase, bone sialoprotein, and osteocalcin. In addition, mineralization of the matrix was blocked in response to breast cancer conditioned medium. TGFβ, present at 5ng/ml, was identified as the factor in the conditioned medium that caused the inhibition in differentiation in maturing osteoblasts.

We further observed that conditioned medium from MDA-MB-231 cells caused both an alteration in osteoblast stress fiber formation and a reduction in focal adhesion plaques [11]. To determine the specificity of the conditioned medium, MC3T3-E1 osteoblasts were also cultured with conditioned medium from NIH-3T3 fibroblasts. Culture with 3T3 fibroblast conditioned medium had no effect on MC3T3-E1 actin stress fiber formation or differentiation. In addition, human primary osteoblasts were cultured with MDA-MB-231 conditioned medium. These cells had altered actin stress fibers as well as a reduction in alkaline phosphatase activity.

4

The aim of this current study was to determine the signaling pathways and molecules used by MDA-MB-231 cells to alter osteoblast stress fiber formation and reduce focal adhesion plaques. Platelet derived growth factor (PDGF), insulin like growth factor II (IGFII) and TGFβ were found to be responsible for these effects. The signaling pathways from each of these molecules merged at phosphatidylinositol 3'-kinase (PI3K) and further signaled through Rac.

MATERIALS AND METHODS

Materials

Culture media, DMEM and α–MEM with glutamine and nucleosides were obtained from MediaTech (Herndon, VA). Fetal bovine serum (FBS), a neutralizing antibody to PDGF (P6101) and LY294002 were purchased from Sigma (St. Louis, MO). Neutralizing antibodies to TGFβ (MAB1835) and IGF-II (MAB292) were purchased from R&D Systems (Minneapolis, MN). RNeasy® mini kit and SuperFect® transfection reagent were purchased from Qiagen (Maryland). AlexaFluor 568 phalloidin was obtained from Molecular Probes (Eugene, OR). All PCR primers were purchased from Integrated DNA technologies (Coralville, IA). QuantumRNA™ 18s Internal Standards were purchased from Ambion (Austin, TX). All plasmids (dominant negative rho, rac, and cdc42, and GFP-expression plasmid) were a kind gift from Dr. Avery August, Penn State University.

Cells and culture conditions

MC3T3-E1 osteoblasts [12] were a gift from Dr. Norman Karin, University of Delaware. They were maintained in α–MEM with glutamine and nucleosides plus 10% FBS. MC3T3-E1 osteoblasts were passaged every 3 to 4 days using 0.002% pronase. MC3T3-E1 cells were not used above passage number 20. MDA-MB-231 cells[13, 14] were a gift from Dr. Danny Welch, University of Alabama at Birmingham, and were maintained in DMEM containing 5% fetal bovine serum (FBS). All cells were cultured in a humidified 37°C incubator containing 5% CO_2 and 95% air.

For production of MDA-MB-231 conditioned medium, cells were cultured to about 90% confluence in T-150 flasks, rinsed twice with phosphate buffered saline (PBS) and cultured with 20ml of serum free α-MEM for 24 hours. The conditioned medium was collected, centrifuged at 1000xg for 10 minutes, pooled, aliquoted, and frozen at –20°C until used.

For all experiments, MC3T3-E1 osteoblasts were initially plated in differentiation medium (α-MEM plus 10% FBS, 50ug/ml ascorbic acid, and 10mM β-glycerophosphate), at a cell density of 10^4 cells/cm^2 and cultured overnight. The following day (unless otherwise stated), medium was replaced with 50% vehicle control medium (serum free α-MEM) or MDA-MB-231 conditioned medium plus 50% 2x differentiation medium (α-MEM + 20% FBS, 100ug/ml ascorbic acid, and 20mM β-glycerophosphate). Cells were cultured the indicated number of days, receiving media changes every other day.

Neutralization of MDA-MB-231 conditioned medium

MDA-MB-231 conditioned medium or vehicle control medium was incubated at 37°C for a minimum of 2 hours with the following antibodies prior to culture with MC3T3-E1 osteoblasts: anti-TGFβ neutralizing antibody was used at a concentration of 10μg/ml, sufficient to neutralize 25ng/ml of TGFβ; anti-PDGF neutralizing antibody was used at

1mg/ml, sufficient to neutralize 20ng/ml of PDGF; anti-IGFII neutralizing antibody was used at 1mg/ml, sufficient to neutralize 7ng/ml of IGFII.

F-actin staining

MC3T3-E1 osteoblasts were plated on Thermanox plastic coverslips and cultured with vehicle control medium or MDA-MB-231 conditioned medium. After 3 days, the cells were fixed in 4% paraformaldehyde, stained with AlexaFluor 568 phalloidin (1:50) and SYBR Green (1:5000) for 20 minutes, and then visualized by confocal microscopy. Three to four fields were examined for each experiment. Each experiment was performed three times. A 1 tailed t-test was performed comparing the number of f-actin clusters in vehicle control treated cells to the number of clusters in conditioned medium treated cells.

Interference reflection microscopy

MC3T3-E1 osteoblasts were plated on fibronectin-coated (20μg/ml) glass coverslips. The following day, medium was switched to either 50% vehicle control medium or MDA-MB-231 conditioned medium, plus 50% 2x differentiation medium. After 3 days, focal adhesion plaques in live cells were visualized by interference reflection microscopy using a Bio-Rad MRC1024 confocal microscope supported by a Dell Power Edge 1600SC with a blue reflection filter. Through this technique, focal adhesion plaques reflect light and are visualized as black spots. Each experiment was

performed three times. Three or four fields were analyzed per experiment. Images were saved as TIF files. Because it was not always possible to distinguish individual cells, focal adhesion plaques were counted per cell area and quantified as plaques per pixel^2 using the program ImageJ 1.32j (http://rsb.info.nih.gov/ij/). A 1 tailed t-test was performed comparing vehicle control treated cells to conditioned medium treated cells.

Transfection of MC3T3-E1 osteoblasts

MC3T3-E1 osteoblasts were plated on Thermanox plastic coverslips at $2x10^4$ cells/cm^2 in 24 well plates in differentiation medium and allowed to incubate overnight. The following day, 1µg of dominant negative plasmid (rac, rho or cdc42) plus 1µg of GFP-expression plasmid was added to serum-free α–MEM to a total volume of 60µl. SuperFect® transfection reagent was added to the DNA and incubated at room temperature for 10 minutes. Medium (350µl of α-MEM plus 10% FBS) was added to the DNA/Superfect mix. MC3T3-E1 osteoblasts were washed with PBS and incubated with the DNA/Superfect mixture at 37°C for 3 hours. The DNA/Superfect mixture was removed and the cells were washed with PBS. The cells were incubated with either 50% vehicle control medium or MDA-MB-231 conditioned medium plus 50% 2x differentiation medium. After 3 days of culture, the cells were prepared for F-actin staining. Each experiment was performed three times; 3-4 fields were analyzed per experiment.

RESULTS

Signaling through PI3K

Confluent cultures of MC3T3-E1 osteoblasts normally form a cobblestone pattern [11, 15]. When cultured to confluence with conditioned medium from MDA-MB-231 cells, the osteoblasts became elongated, more fibroblast-like, and appeared to no longer be in a monolayer (figure 1). However, when MC3T3-E1 osteoblasts were treated with LY294002, a PI3K inhibitor, and breast cancer conditioned medium, the pattern resembled that of control, non-treated cells, indicating that the breast cancer-induced alterations in morphology were mediated through PI3K.

We next asked if MDA-MB-231 conditioned medium also altered actin stress fibers through a PI3K mediated pathway. MC3T3-E1 osteoblasts were cultured with MDA-MB-231 conditioned medium with or without LY294002. Cells cultured in vehicle control medium with LY294002 displayed strong actin stress fiber formation, while cells cultured with MDA-MB-231 conditioned medium showed a reorganization of f-actin. The f-actin in these cells was largely cortical, or accumulated in large clusters, rather than stretching across the cells as stress fibers. When MC3T3-E1 osteoblasts were treated with MDA-MB-231 conditioned medium and LY294002, actin stress fiber formation was stronger, resembling that of vehicle control treated cells (figure 2). To quantify the changes in actin organization, the number of clustered actin per cell was counted. While cells treated with MDA-MB-231 conditioned medium had a significant

increase in clustered actin (p<0.00005), cells treated with MDA-MB-231 conditioned medium plus LY294002 resembled controls.

Because PI3K signals through many pathways, we wanted to determine specifically which pathway MDA-MB-231 conditioned medium induced in MC3T3-E1 osteoblasts. To do this, the small G-proteins rac, rho and cdc42, each located downstream of PI3K, were assayed. Dominant negative plasmids for rac, rho and cdc42 were co-transfected into MC3T3-E1 osteoblasts along with a GFP expression plasmid. The osteoblasts were then treated with MDA-MB-231 conditioned medium and the GFP positive cells were assayed for actin stress fiber formation. Osteoblasts transfected with dominant negative rac and treated with MDA-MB-231 conditioned medium exhibited stress fiber patterns not significantly different from vehicle control cells (figure 3). On the other hand, neither dominant negative rho nor dominant negative cdc42 reversed the alterations induced by MDA-MB-231 conditioned medium.

In addition to alterations in actin stress fibers, we examined the presence of focal adhesion plaques through interference reflection microscopy. MC3T3-E1 osteoblasts were cultured with MDA-MB-231 conditioned medium with or without LY294002. Cells cultured with MDA-MB-231 conditioned medium showed a significant (40%) reduction in focal adhesion plaques compared to cells treated with vehicle control medium (p<0.05). Those cultured with breast cancer conditioned medium and LY294002 had a complete restoration of focal adhesion plaques (figure 4) compared to cells treated with vehicle control medium.

Cytokines

MDA-MB-231 conditioned medium has been reported to contain cytokines known to signal through PI3K, including TGFβ [11, 16], PDGF [17], and IGFII [16]. MC3T3-E1 osteoblasts were cultured with MDA-MB-231 conditioned medium pre-treated with neutralizing antibodies against TGFβ, PDGF or IGFII. Neutralization of either TGFβ or PDGF restored MC3T3-E1 morphology to that of a patterned, cobblestone appearance. Neutralization of IGFII from MDA-MB-231 conditioned medium had no effect on MC3T3-E1 morphology (figure 5).

These three cytokines were also examined for their role in MDA-MB-231 induced changes in actin stress fiber formation. Neutralization of TGFβ, PDGF, or IGFII singly did not reverse the reorganization f-actin caused by MDA-MB-231 conditioned medium (data not shown). Therefore, the cytokines were neutralized in combination. A full recovery in actin staining from cortical, clustered staining to strong stress fiber formation was only observed when all three cytokines in the conditioned medium were neutralized (figure 6).

In accordance with these observations, neutralized conditioned medium was assayed for its effects on MC3T3-E1 osteoblast focal adhesion plaques. Neutralizing each cytokine singly had no effect on restoration of focal adhesion plaques (data not shown). Neutralization of all three cytokines, TGFβ, PDGF and IGFII, from MDA-MB-231 conditioned medium resulted in an 80% recovery in focal adhesion plaques (figure 7).

DISCUSSION

The focus of this study was to determine how MDA-MB-231 conditioned medium reduced osteoblast focal adhesion plaques and altered the f-actin expression pattern. Using RT-PCR to analyze mRNA levels of actin, we found no differences between vehicle control and conditioned medium treated cells (data not shown). Therefore, we believe the changes in f-actin are due to a reorganization of actin stress fibers rather than a reduction in expression levels. We found that TGFβ, IGFII, and PDGF present in MDA-MB-231 conditioned medium caused these effects in MC3T3-E1 osteoblasts, and that they signal through PI3K and rac.

We demonstrated that PDGF present in MDA-MB-231 conditioned medium affected the normal f-actin pattern in osteoblasts as well as reduced the number of focal adhesion plaques. Initially isolated from platelets, PDGF naturally occurs in bone. It is synthesized by osteoblasts, and synthesis can be upregulated by TGFβ. The primary function of PDGF in bone cells is to enhance mitogenic activity of osteoblasts. PDGF can also indirectly stimulate bone resorption and collagen degradation through the upregulation of matrix metalloproteinases MMP1 and MMP3 [18].

In addition to its mitogenic potential in osteoblasts, PDGF has also been shown to induce chemotaxis in other cell types, such as fibroblasts, smooth muscle cells and phagocytic cells. Interestingly, PDGF can cause the reorganization of actin filaments, inducing membrane ruffling [19]. To induce actin reorganization, a PDGF dimer first binds two PDGF receptors simultaneously, leading to receptor dimerization. Upon dimerization, tyrosines in the receptor become cross- and auto-phosphorylated, providing

docking sites in the cytoplasm for SH_2 containing proteins. PI3K can then bind the activated receptor. PI3K has many downstream effector molecules and plays a central role in signal transduction for many cellular responses [19]. To induce actin reorganization, the small G-protein, rac, is activated [20]. Rac then stimulates the uncapping of f-actin from the barbed end through the phosphoinositide P1(4,5)P2 [21].

Perhaps in coordination with actin reorganization, PDGF also disassembles focal adhesion plaques [22]. Actin stress fibers combine at sites of adhesion, creating focal adhesion plaques. These plaques contain many proteins, such as talin, vinculin, and paxillin, that colocalize at sites of adhesion. Upon binding through β_1 integrin, various adhesion proteins are phosphorylated and connect actin stress fibers to the β_1 integrin. A survival signal is subsequently sent to the nucleus. The PDGF stimulus leads to a disassembly of β_1 integrins from focal adhesion plaques to a random distribution throughout the periphery of the cells, thus temporarily disrupting focal adhesion plaques. This process is mediated through PI3K [22].

In this study, we further demonstrated that IGFII, present in MDA-MB-231 conditioned medium, altered osteoblast stress fiber formation and reduced focal adhesion plaques. The primary role of IGFII is to aid in embryonic and fetal development, while IGFI is most important in postnatal development. IGFs are normally found bound in a ternary complex with IGF binding proteins (IGFBPs) and the acid-labile subunit (ALS). IGFBPs determine the bioavailability of IGF by sequestering it in the bloodstream and allowing for its travel through capillary beds to the target cells. IGF is slowly released from the IGFBPs by local IGFBP proteases [23].

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Both IGFI and IGFII promote cellular growth and stimulate insulin-like metabolic activity [24]. Both molecules signal through the heterotetrameric tyrosine kinase receptor, IGF-IR. Binding of IGF to the α subunits results in dimerization of the protein. The β subunits then phosphorylate each other and the docking molecule insulin-receptor substrate (IRS). IRS contains tyrosine phosphorylation sites that serve as binding sites for PI3K [25]. PI3K can then signal through rac, leading to membrane ruffling and actin reorganization [26].

While we previously reported that TGFβ in MDA-MB-231 conditioned medium inhibited differentiation in maturing osteoblasts, this study indicates that TGFβ also contributes to alterations in immature osteoblasts. TGFβ is an important modulator of osteoblast function. Active TGFβ dimerizes and binds to osteoblasts through the type II receptors. Binding to the type II receptor facilitates dimerization with the type I receptor, which can then initiate the SMAD pathway. Initiation of this pathway is thought to inhibit osteoblast differentiation. Activated phospho-SMAD3 will bind SMAD4 and cross into the nucleus. This complex binds DNA and regulates transcription. One transcriptional target of the SMAD complex is Runx2. The SMAD complex directly interacts with Runx2, although this interaction does not displace Runx2 from the DNA. Instead, it appears to enhance Runx2 binding to the DNA. SMAD-3 bound to Runx2 reduces transcriptional activity of the promoter. This specifically occurs at the Runx2 binding site, OSE1, present both in the osteocalcin and alkaline phosphatase promoters [27].

Although signaling through SMAD3 is the classical TGFβ pathway, others have found that TGFβ can also signal through PI3K and the MAPK pathways [28, 29].

Signaling through PI3K leads to changes associated with cell adhesion, such as induction of epithelial to mesenchymal transition (EMT), dislocalization of both E-cadherin and β_1 integrin, and a spindle-cell morphology [30, 31]. We propose that by signaling through PI3K, TGFβ is in part contributing to the alterations in stress fiber formation and the reduction in focal adhesion plaques.

PDGF, IGF and TGFβ have all been shown to signal through PI3K. Rac is activated downstream of PI3K, leading to reorganization of actin filaments, which can alter the prevalence of focal adhesion plaques. In addition, PI3K signaling leads to dislocalization of β_1 integrin, also leading to a reduction in focal adhesion plaques. In our study, the data indicated that these three cytokines in combination contributed to the reorganization of f-actin stress fibers and the reduction in focal adhesion plaques induced by MDA-MB-231 conditioned medium.

Previously, using scanning acoustic microscopy, we found that MDA-MB-231 conditioned medium reduced MC3T3-E1 attachment [11]. The reduction in attachment correlated with changes in f-actin stress fiber formation and the number of focal adhesion plaques. These data suggest that MDA-MB-231 conditioned medium alters osteoblast adhesion, although this has not yet conclusively been shown. MC3T3-E1 osteoblasts plated directly in 50% MDA-MB-231 conditioned medium do not display the same phenotype of those cells cultured with conditioned medium 24 hours after plating. Rather, osteoblasts plated in conditioned medium die within 24 hours of attachment (data not shown), making adhesion experiments difficult to perform and interpret.

Cell adhesion is important in the osteoblast life cycle, particularly during the transition from the growth phase to the beginning stages of matrix synthesis. In order for

differentiation to proceed, osteoblasts must bind and signal through $\alpha_2\beta_1$ integrin. Osteoblasts express a variety of integrins; however, β_1 integrin is dominant. The $\alpha_2\beta_1$ integrin binds to collagen secreted by proliferating osteoblasts, resulting in the upregulation of Runx2, alkaline phosphatase, and osteocalcin [32-34]. The importance of adhesion is further indicated in a mouse model that lacked β_1 integrin. The animals had decreased bone mass and an osteoporotic phenotype, indicating that adhesion is paramount to osteoblast function [35].

Future studies will determine if the altered actin expression pattern and reduced number of focal adhesion plaques affect osteoblast differentiation. While we found that TGFβ present in MDA-MB-231 conditioned medium inhibited osteoblast differentiation [11], these studies were performed with maturing osteoblasts (10 days old). The cells were then cultured for an additional 6 days (16 days total) before they were assayed for differentiation. Because the MC3T3-E1 osteoblasts were cultured 10 days prior to the addition of MDA-MB-231 conditioned medium, this experiment bypassed the alterations in actin and focal adhesion plaques that conditioned medium induced in early, immature osteoblasts.

The scientific community has largely focused on understanding the role of the osteoclast in breast cancer skeletal metastasis. We propose that communication between breast cancer cells and osteoblasts is equally relevant in understanding this complex disease. We now know that breast cancer cells induce osteoblast apoptosis [9, 10], inhibit osteoblast differentiation [11, 36], and alter osteoblast attachment [11]. Collectively, these data indicate that the osteoblast is gravely affected in lytic metastases.

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In order to fully treat this disease, the communication between breast cancer cells and osteoblasts must also be considered.

Figure Legends

Figure 1. The PI3K inhibitor LY294002 restored the cobblestone morphology of MC3T3-E1 osteoblasts when cultured with MDA-MB-231 conditioned medium. MC3T3-E1 osteoblasts were cultured with 50% MDA-MB-231 conditioned medium (231 CM) or vehicle control medium (VM) plus 10μM LY294002 for 5 days. The cells were stained with H&E and viewed through a bright field microscope. A: vehicle control medium; B: vehicle control medium plus LY294002; C: MDA-MB-231 conditioned medium; D: MDA-MB-231 conditioned medium plus LY294002. This experiment was performed 3 times with the same results.

Figure 2. LY294004 reversed the effect of MDA-MB-231 conditioned medium on F-actin organization in MC3T3-E1 osteoblasts. MC3T3-E1 osteoblasts were cultured with either 50% MDA-MB-231 conditioned medium or 50% vehicle control medium plus 10μM LY294002 for 3 days. F-actin was stained with phalloidin and cell nuclei were visualized with SYBR Green. A: vehicle control medium; B: vehicle control medium plus LY294002; C: MDA-MB-231 conditioned medium; D: MDA-MB-231 conditioned medium plus LY294002. E: Changes in the f-actin pattern were quantified by counting the number of actin clusters per cell. Shown are the averages, +/- standard deviation, of 3 experiments; 3 fields were analyzed per experiment. A 1 tailed t-test was used to compare all values to the average value of vehicle control medium. * p-value <0.00005. VM: vehicle control medium; VM + LY: vehicle control medium plus LY294002; CM:

MDA-MB-231 conditioned medium; CM + LY: MDA-MB-231 conditioned medium plus LY294002.

Figure 3. MDA-MB-231 conditioned medium signals through the small G-protein rac. MC3T3-E1 osteoblasts were co-transfected with a GFP-expression plasmid plus a dominant negative plasmid for rac, rho, or cdc42. Following transfection, osteoblasts were cultured with either 50% MDA-MB-231 conditioned medium or 50% vehicle control medium. F-actin was visualized by phalloidin staining. A, C, E, G: vehicle control medium; B, D, F, H: MDA-MB-231 conditioned medium; A and B: GFP plasmid only; C and D: GFP plasmid plus dominant negative rac; E and F: GFP plasmid plus dominant negative rho; G and H: GFP plasmid plus dominant negative cdc42. I: Changes in the f-actin pattern were quantified by counting the number of actin clutsers per cell. Shown are the averages, +/- standard deviation, of 3 experiments; 3 fields were analyzed per experiment. A 1 tailed t-test was used to compare all values to the average value of vehicle control medium. * p-value <0.0005.

Figure 4. MDA-MB-231 conditioned medium reduced focal adhesion plaques in MC3T3-E1 osteoblasts through PI3K. MC3T3-E1 osteoblasts were cultured with 50% MDA-MB-231 conditioned medium or 50% vehicle control medium plus 10µM LY294002 for 3 days on fibronectin coated glass coverslips. Focal adhesion plaques were visualized in live cells using interference reflection microscopy. A: vehicle control medium; B: vehicle control medium plus LY294002; C: MDA-MB-231 conditioned medium; D: MDA-MB-231 conditioned medium plus LY294002. Arrows indicate focal

adhesion plaques. E: Focal adhesion plaques were quantified by counting the number of

plaques per cell area. Shown are the averages, +/- standard deviation, of 3 experiments; 3

fields were analyzed per experiment. A 1 tailed t-test was used to compare all values to

the average value of vehicle control medium. * p-value <0.05. VM: vehicle control

medium; VM + LY: vehicle control medium plus LY294002; CM: MDA-MB-231

conditioned medium; CM + LY: MDA-MB-231 conditioned medium plus LY294002.

Figure 5. PDGF and TGFβ, present in MDA-MB-231 conditioned medium, altered the

morphology of MC3T3-E1 osteoblasts. MDA-MB-231 conditioned medium or vehicle

control medium was incubated with antibodies to neutralize PDGF, IGF, or TGFβ and

cultured with MC3T3-E1 osteoblasts for 4 days. A, C, E, G: vehicle control medium; B,

D, F, H: MDA-MB-231 conditioned medium; A and B: no antibodies; C and D: media

plus anti-PDGF; E and F: media plus anti-IGF; G and H: media plus anti-TGFβ. This

experiment was performed 3 times.

Figure 6. F-actin organization was restored in MC3T3-E1 osteoblasts cultured with

MDA-MB-231 conditioned medium neutralized for PDGF, IGF and TGFβ. Vehicle

control medium or MDA-MB-231 conditioned medium was pre-incubated with the

antibodies to neutralize PDGF, IGF and TGFβ in combination. After neutralization,

MC3T3-E1 osteoblasts were incubated with the media for 3 days and then assayed for F-

actin using phalloidin and cell nuclei with SYBR Green. A: vehicle control medium; B:

vehicle control medium pre-incubated with the neutralizing antibodies against PDGF,

IGF, and TGFβ; C: MDA-MB-231 conditioned medium; D: MDA-MB-231 conditioned

medium pre-incubated with the neutralizing antibodies against PDGF, IGF, and TGFβ.

E: Changes in the f-actin pattern were quantified by counting the number of actin clusters

per cell. Shown are the averages, +/- standard deviation, of 3 experiments; 3 fields were

analyzed per experiment. A 1 tailed t-test was used to compare all values to the average

value of vehicle control medium. * p-value <0.01.

Figure 7. Neutralization of PDGF, IGF and TGFβ from MDA-MB-231 conditioned

medium restored focal adhesion plaques in MC3T3-E1 osteoblasts. Vehicle control

medium (VM) or MDA-MB-231 conditioned medium (231 CM) was neutralized for

PDGF, IGF and TGFβ and incubated with MC3T3-E1s for 3 days. Focal adhesion

plaques were visualized using interference reflection microscopy. A and B: vehicle

control medium; C and D: MDA-MB-231 conditioned medium; A and C: no antibodies;

B and D: medium neutralized for PDGF, IGF, and TGFβ. Arrows indicate focal

adhesion plaques. E: Focal adhesion plaques were quantified by counting the number of

plaques per cell area using ImageJ 1.32j. Shown are the averages, +/- standard deviation,

of 3 experiments; 3 fields were analyzed per experiment. A 1 tailed t-test was used to

compare all values to the average value of vehicle control medium. * p-value <0.01

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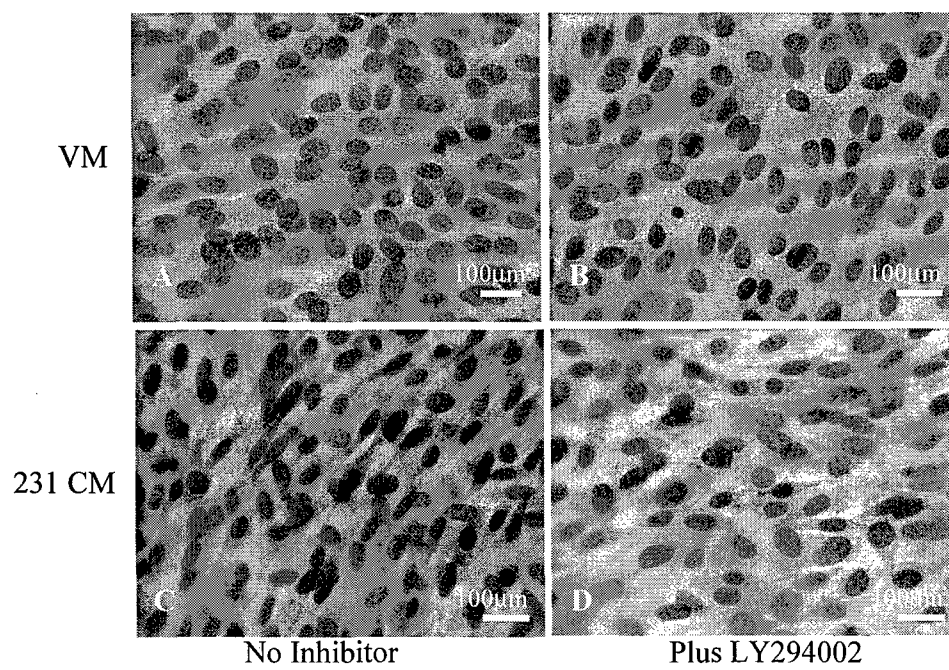
FIGURE 1

VM

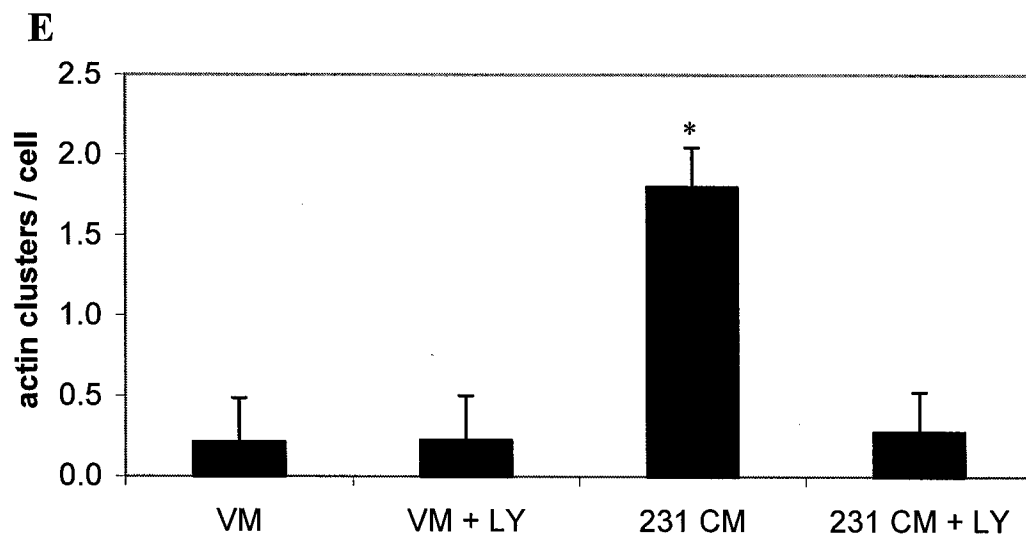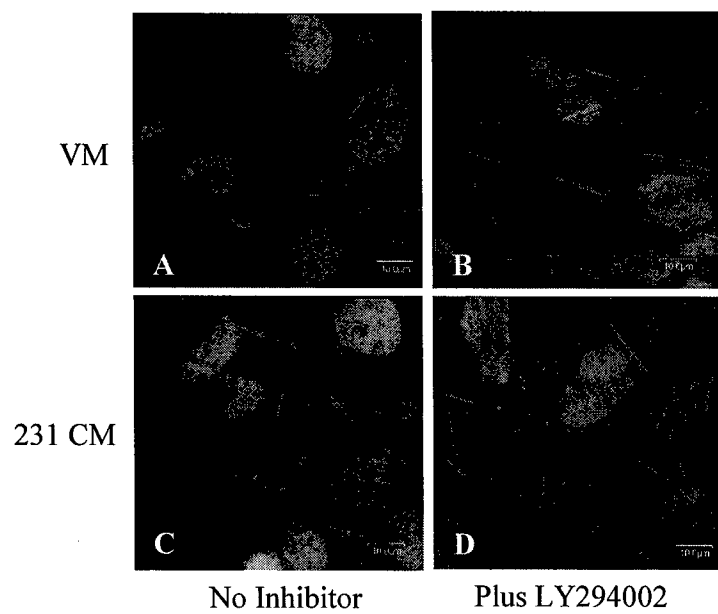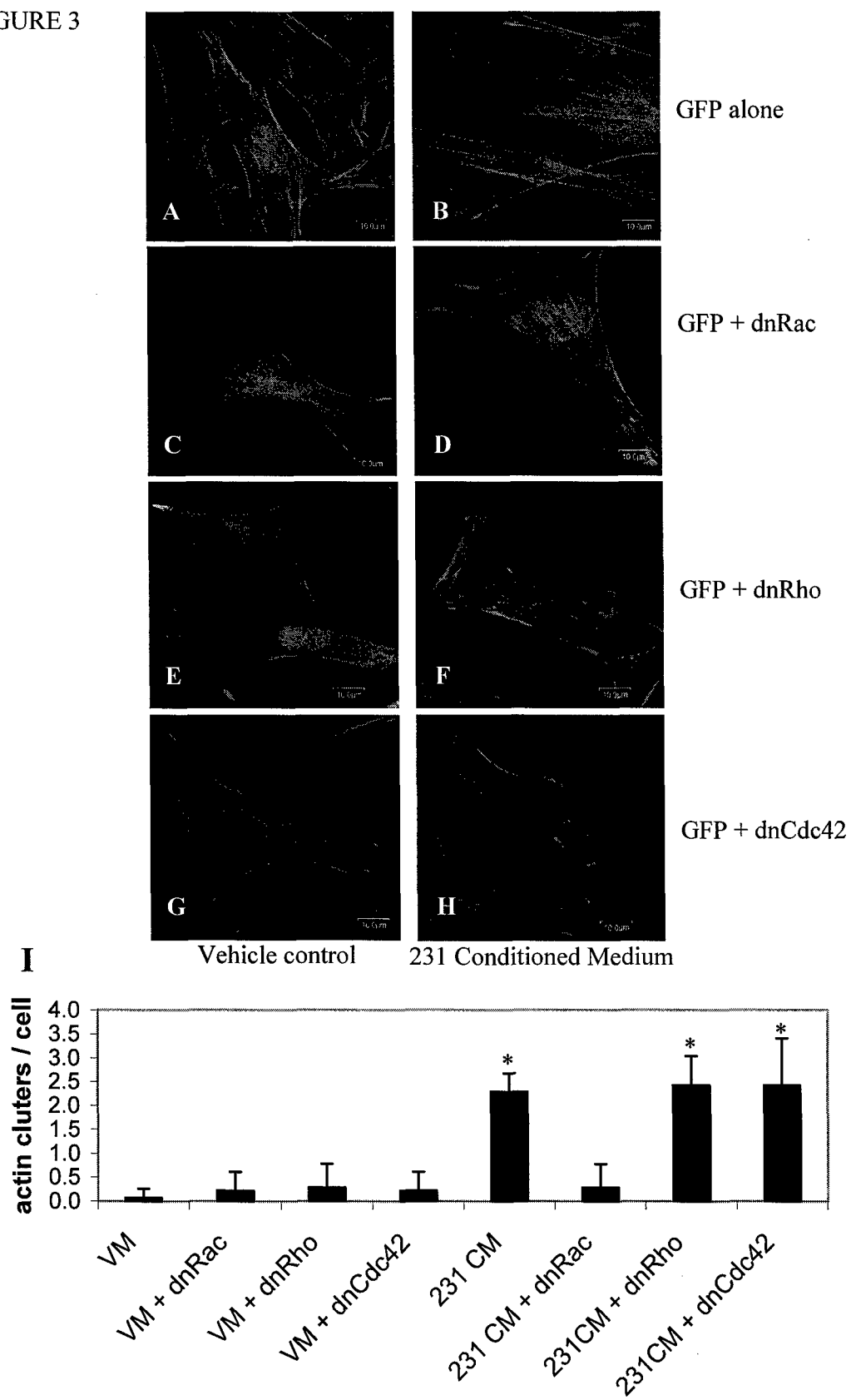231 CM

No Inhibitor   Plus LY294002

FIGURE 2

VM

231 CM

No Inhibitor   Plus LY294002

E

FIGURE 3

GFP alone

GFP + dnRac

GFP + dnRho

GFP + dnCdc42

Vehicle control | 231 Conditioned Medium

FIGURE 4

VM

231 CM

No Inhibitor　　　　Plus LY294002

E

plaques/ cell area (x 10^4)

VM　　VM + LY　　CM　　CM + LY

FIGURE 5

VM   231 CM

No antibodies

Plus anti-PDGF

Plus anti-IGFII

Plus anti-TGFβ

FIGURE 6

	No antibodies	Plus anti-PDGF, anti-IGF, and anti-TGFβ
VM	A	B
231 CM	C	D

FIGURE 7

VM

231 CM

no antibodies | Plus anti-TGFβ, anti-PDGF, and anti-IGF

E

plaques / cell area (x10^4)