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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

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 is to understand what happens to osteoblasts in the presence of breast cancer. We hypothesize that breast cancer cells prevent pre-osteoblasts from completely maturing to osteoblasts. Our goals are 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 upregulated osteoblast proliferation, inhibited alkaline phosphatase activity, and blocked mineralization. Over the next two years, we will continue to examine the cause of these effects in detail.

By understanding the effects of breast cancer on osteoblast differentiation, we will gain insight into how breast cancer cells alter osteoblast function. With this information, new drugs or therapies can be developed to activate osteoblasts in order to heal the bone lesions.

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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 hypothesize that breast cancer cells alter the ability of osteoblasts to differentiate into mature, functional, matrix-producing cells. The aims of this proposal are 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 bone-metastatic breast cancer cell lines MDA-MB-231 and MDA-MB-435 cells were 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

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)

hFOBs are a human fetal osteoblast cell line that can differentiate in culture. These cells were cultured with conditioned medium from MDA-MB-231 and MDA-MB-435 cells. 50% conditioned medium or 50% vehicle control medium was added to hFOBs either at the time of plating or after 2 or 4 days. hFOB cell number was determined indirectly by analyzing mitochondrial activity with the MTT assay. The MTT reagent is a substrate for mitochondrial dehydrogenase. Once added to the cells, it penetrates the cell membrane and is cleaved into an insoluble purple crystal, which is solublized by adding an acidic solution containing Triton X-100. This solution is analyzed spectrophotometrically. Using this procedure, we found that both MDA-MB-231 and MDA-MB-435 conditioned media upregulated hFOB proliferation (Figure 1). Adding the conditioned medium at the time of plating had the greatest effect on hFOB proliferation.

This experiment was also performed with another osteoblast cell line, MC3T3-E1. These cells are an immature mouse osteoblast cell line that will also differentiate in culture. MDA-MB-231 conditioned medium were added to these cells 1 day after plating. Proliferation also increased with this osteoblast cell line.

b. Using the information from task 1a, establish a transwell system to study the effects of breast cancer cell secretions on the hFOB growth cycle. (months 2-4)

A transwell system was established using CoStar transwell inserts such that breast cancer cells were plated inside the insert and hFOBs were plated in the well of a tissue culture plate. As a control, an insert without breast cancer cells was added to a well

containing hFOBs. After 2 days of culture, the hFOBs began to die. We hypothesize that the addition of the transwell insert altered either the pH or oxygen tension, leading to cell death. We plan to try these experiments again with alterations in medium volume and size of the transwell.

c. ***Repeat task 1a. using direct co-culture. All cells will be stained with DAPI and counted with fluorescence microscopy to distinguish GFP expressing breast cancer cells from non-fluorescent hFOB cells. (months 5-6)***

While these experiments have not yet been performed, we have demonstrated that breast cancer cells can be distinguished from osteoblasts by staining with either DAPI or sytox orange and then examining for GFP expression.

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

These experiments will be laborious and expensive. We will not use primary cells until we have obtained sufficient data to warrant their use.

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)***

The goal was to analyze mRNA production using quantitative RT-PCR. I began this project with very little laboratory experience, and no experience with PCR. It took approximately 6 months to optimize the protocol for quantitative RT-PCR. Even with an optimized protocol, large amounts of error were observed within replicate samples of hFOBs. Furthermore, the data were not repeatable between experiments. This was a problem not only for the mRNA expression, but also protein production, as determined by western blotting. Alkaline phoshatase protein production was examined and found in one experiment to be downregulated in response to conditioned medium and upregulated in another, identical experiment. We speculated that the problems in consistency were due to heterogeneity in the cell culture, rather than to technique, for reasons described below.

The hFOB cell line is transformed with a temperature sensitive SV40 large T antigen. This antigen is expressed at 34°C and degraded at 39°C. Large T antigen will bind to p53, allowing cell proliferation at the permissive temperature. At the restrictive temperature, large T antigen is degraded, causing an upregulation of p53. Proliferation will then slow and the cells will begin to differentiate.

In our hands, the hFOBs would only moderately slow their growth at the restrictive temperature, and after a few days at 39°C they often peeled off the substrate. We proposed that the lack of reproducibility observed with the RT-PCR and western blots may have been due to the cells not being synchronous at 39°C. If this were the case, some cells may have slowed their growth and began to differentiate, while others may have continued to proliferate and remained immature. To correct this problem, the cells were

plated and cultured directly at 39°C instead of culturing first at 34°C to confluence, and then switching to 39°C. We thought this would prevent the cells from expressing large T antigen, thus slowing their proliferation. While this did help, the cells still continued to proliferate at 39°C. Eventually, we found that decreasing the amount of serum in the media slowed their proliferation. To prove that the cells could still differentiate under these conditions, quantitative RT-PCR was performed for BSP and OCN expression, an early and late marker of differentiation. Peaks in expression were observed for both proteins. Using the new cell culture conditions for hFOBs, conditioned medium from MDA-MB-231 cells were added to the osteoblasts on the day of plating. mRNA was isolated and subjected to quantitative RT-PCR for both alkaline phosphatase and osteocalcin. Unfortunately, large amounts of error were still observed.

Due to the complications observed with the hFOB cell line, we decided to examine another osteoblast cell line, MC3T3-E1, a line that is not transformed. Because this cell line slows proliferation after confluence, we believe that it will provide more consistent data. These experiments are currently in progress. Preliminary data are promising.

Tasks 2b-2h: These experiments have not yet been addressed due to the complications already described.

In addition to the outlined tasks, the MC3T3-E1 cell line was assayed for alkaline phosphatase activity in the presence of MDA-MB-231 conditioned medium. The cells were cultured with 50% conditioned medium from either MDA-MB-231 cells or NIH 3T3 fibroblasts, or with 50% vehicle control medium for 35 days. NIH 3T3 fibroblast conditioned media were used as a negative control. To assay for alkaline phosphatase activity, the cells were fixed in formaldehyde and stained with Napthol AS-BI Phosphate and Fast Blue RR Salt. This solution provides a substrate for alkaline phosphatase that when cleaved, turns blue. MC3T3-E1 cells had no detectable alkaline phosphatase activity when cultured with MDA-MB-231 cell conditioned media, but activity was not affected by NIH 3T3 fibroblast conditioned media or vehicle control media (Figure 2).

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)

We have not been able to show mineralization of the hFOB cell line through Von Kossa staining. However, MC3T3-E1 cells do mineralize in culture and stain with Von Kossa. 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) (data not shown).

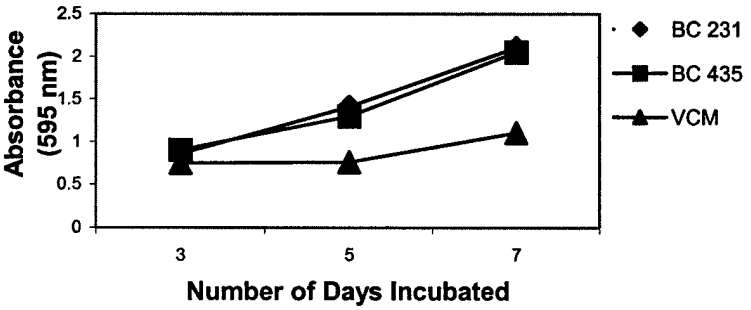
Tasks 3b-3c: These tasks have not yet been addressed. The MC3T3-E1 cell line is currently being assayed for alterations in mRNA expression of osteocalcin in response to MDA-MB-231 conditioned medium.

Task 4: Data analysis and thesis preparation. Task 4 has not been addressed.

In addition to the tasks outlined above, we have made further observations with the MC3T3-E1 cell line. When initially culturing these cells to determine MDA-MB-231 conditioned medium effects on mineralization, we noticed that the conditioned medium induced a change in cell morphology. Once MC3T3-E1 cells reach confluence, they lose their fibroblast morphology and begin to take on a patterned, cobblestone appearance. However, in MDA-MB-231 conditioned medium, the cells become very long and spindle shaped. Based on these changes in morphology, we looked at the actin filaments in the cells. MC3T3-E1 cells were cultured with MDA-MB-231 conditioned medium for 9 days, fixed with paraformaldehyde, and stained with phalloidin to identify f-actin. Stress fiber formation was evident in the control cells, but only cortical staining was observed in the cells treated with conditioned medium (Figure 3). We suspect that these changes in stress fiber formation may be upstream of the observed defects in alkaline phosphatase production and mineralization.

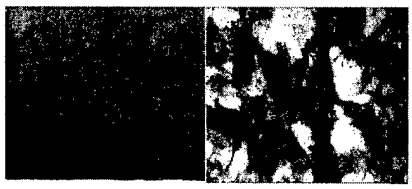
Progress in Program: From the time this proposal was submitted, all required course work for a PhD. has been completed. In October of 2001, I passed the candidacy examination; and in October of 2002, I successfully completed the comprehensive examination. I was recently selected to attend the "Pathobiology of Cancer Workshop" sponsored by the American Association for Cancer Research, to be held in July, 2003.

Figure 1: Breast Cancer Conditioned Media Enhances hFOB Proliferation



MDA-MB-231 or MDA-MB-435 conditioned medium, or vehicle control medium (vcm) were added to hFOBs 4 hours after plating. Cell number was determined using the MTT assay. Results are representative of triplicate samples.

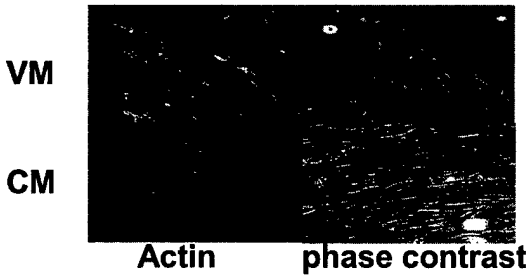
Figure 2: MDA-MB-231 Conditioned Medium inhibits Alkaline Phosphatase Activity



231 conditioned medium | vehicle control medium

MC3T3-E1 cells were cultured with conditioned medium from MDA-MB-231 cells for 35 days and then stained for alkaline phosphatase activity. (40x magnification)

Figure 3: MDA-MB-231 Conditioned Medium prevents stress fiber formation in MC3T3-E1 cells



MC3T3-E1 cells were cultured with conditioned medium from MDA-MB-231 cells and stained with phalloidin. Stress fibers are evident in vehicle control samples (VM), but not in the conditioned medium treated samples (CM). (40x magnification)

Key Research Accomplishments:

- Established a protocol for quantitative RT-PCR
- Characterized a second osteoblast culture system
- Made many observations with osteoblasts cultured with cancer conditioned medium

Reportable Outcomes: **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 (in press 2003)

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.

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. Thus far, we have learned that breast cancer cells alter the proliferation rate of immature osteoblasts and affect their differentiation pattern. We experienced many problems with the hFOB cell line and thus will continue to use MC3T3-E1 cells in the future. In addition, we have made many observations when culturing MC3T3-E1 cells with MDA-MB-231 conditioned medium, but due to page limitations can not report all of them here. Based on these new observations, a revised Statement of Work will be submitted.

References: None
Appendices: None