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Award Number: DAMD17-03-1-0584

TITLE: Trafficking of Metastatic Breast Cancer Cells in Bone

PRINCIPAL INVESTIGATOR: Andrea M. Mastro, Ph.D.

CONTRACTING ORGANIZATION: Pennsylvania State University
University Park, Pennsylvania 16802-4400

REPORT DATE: August 2004

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2004	Annual (1 Aug 2003 - 31 Jul 2004

4. TITLE AND SUBTITLE
Trafficking of Metastatic Breast Cancer Cells in Bone

5. FUNDING NUMBERS
DAMD17-03-1-0584)

6. AUTHOR(S)
Andrea M. Mastro, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Pennsylvania State University
University Park, Pennsylvania 16802-4400

E-Mail: a36@PSU.EDU

8. PERFORMING ORGANIZATION REPORT NUMBER

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

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

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

Breast cancer cells frequently metastasize to the bone where they grow and destroy bone. Their growth is not random; i.e. tumors are usually found at the metaphyseal ends of long bones rather than in the cortical shaft. The objective of this study is to determine the traffic patterns of breast cancer cells in the marrow cavity and to identify factors that attract them to the metaphyses. Human breast cancer cells that express green fluorescent protein (GFP-MDA-MB 231) will be inoculated into athymic mice by intracardiac injection and femurs harvested at various times from 1 hour to 6 weeks later. Marrow cells will be collected from the proximal and distal metaphyseal ends and from the cortical shaft, and the cancer cells detected by flow cytometry. Histological analysis of femurs will also be carried out. In the second part of the study, samples of metaphyseal marrow, cortical marrow, metaphyseal bone and cortical bone will be tested for chemotaxis of cancer cells using transwell chambers. We predict that cancer cells follow a definable pathway from entry to where they grow in the bone metaphyses. This information will provide targets to disrupt the localization and the growth of breast cancer cells in the bone.

14. SUBJECT TERMS
breast cancer, bone, metastasis, trafficking

15. NUMBER OF PAGES
9

16. PRICE CODE

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

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INTRODUCTION

Breast cancer cells frequently metastasize to bone where they cause osteolytic lesions. The growth of cancer cells in bone is not random; i.e. tumor masses are usually found at the metaphyseal ends of long bones. However, the basic biology of this event is not understood (Mundy 2002). For example, it is not known how breast cancer cells arrive at their destination and what attracts them to this site. The objective of this study was to determine the traffic patterns of breast cancer cells in the bone marrow cavity and to identify factors that attract the cells to the metaphyseal ends of the bone. The development of green fluorescent protein (GFP)-expressing breast cancer cells will allow us to detect metastases prior to bone loss (Harms et al. 2002). Athymic mice will be inoculated with GFP-human metastatic breast cancer cells by intracardiac injection and the femurs harvested at various times. The marrow will be collected from the distal, proximal and shaft (central and endosteal) areas of the bone marrow cavity. Histological analysis of fixed bone will also be used to detect the presence of breast cancer cells (Harms et al. 2002). In addition marrow and bone from the same areas of the bone will be cultured. The culture preparations will be tested for chemotaxis of the breast cancer cells. The culture medium also will be screened for the presence of cytokines (Muller et al. 2001).

BODY

The first task was to determine how metastatic breast cancer cells partition among the metaphyseal, endosteal and cortical bone marrows (Mason et al. 1989). We extended the study to examine separately the proximal (hip) metaphyseal region versus the distal (knee) metaphyseal regions of the femur. In the first experiment athymic mice were injected by intracardiac injection (approximately $3x10^5$ breast cancer cells). At various times, (1 hr, 2 hr, 4 hr, 8 hr, 24 hr, 72 hr, 1 week, 2 weeks, 4 weeks, 6 weeks) the femurs were harvested. In order to carry out flow cytometric analysis, marrow was collected from the four regions of the bone: distal metaphyses, proximal metaphyses, diaphysis endosteal marrow and diaphysis central marrow. Femurs for this procedure were taken from 3-9 animals per group (groups: 1 hr, 4 hr, 24 hr, 72 hr, 1 week, 4 weeks, 6 weeks). The red blood cells were lysed from the bone marrow preparations. A number of standard fluorescent beads of known concentration were added to each sample in order to quantitate the numbers of GFP positive breast cancer cells in each preparation. We detected GFP positive cells very early in the marrow of the diaphysis. These probably represented the first pass of the cells through the body. After that we did not detect cells again until 72 hours after

inoculation and this was only in one animal. These cells were also in the central diaphyseal marrow and may have still been circulating cells. We saw no cells at one week in femurs from any animals, but by 4 weeks they were plentiful. In the femurs from all of the mice there were more cells in the distal metaphysis (see Fig 1) than in the other regions. The relative rankings were distal>proximal>central diaphysis>endosteal diaphysis.

Another set of femurs was collected at all the times (4-9 per time) for histology. These bones were fixed, decalcified and embedded in paraffin. A group of control animals, without breast cancer cells, also was included. By four to six weeks the tumors could be seen in the bones by visual inspection; i.e. the are of the bone filled with cancer cells appeared to be white while the area into which the marrow cells were crowded, was brownish in color. One six-week bone was used to establish the procedures and served as a positive control. We determined: 1) GFP-breast cancer cells were detectable in the bone sections (Figure 2); and 2) an anti-GFP peptide antibody (BD Biosciences) combined with a Cy 3 secondary antibody could also be used to detect the GFP breast cancer cells. However, the numbers using the antibody approach were comparable to detection of GFP alone. Therefore, we used quantitated the GFP –positive cells. Each bone was longitudinally sectioned (~ 200 sections) and sections selected from several areas throughout the bone. The sections were examined by confocal microscopy and the images were captured. The fluorescent images were overlayed onto the phase microscopy images of the same sections in order to verify the position of the cancer cells in the bone sections. The numbers of GFP positive cells were counted in 6 to 12 fields per section (Figure 3). These studies are still in progress. However, in agreement with the flow cytometric analysis, the histology studies indicate that the cancer cells traffic to the distal ends of the femurs before they move to the proximal ends (Table 1).

The second task was to compare the chemotactic properties of metaphyseal bone and marrow with endosteal and cortical bone and marrow. The three parts of this task were to prepare bone and marrow cultures; assay breast cancer cells for chemotaxis to the various bone and marrow preparations and assay culture medium from the bone marrow and bone preparations for cytokines and chemokines. We plan to carry-out these tasks in the second year of the project.

There are two reasons for the delay in this project. One, the first task, especially the

immunohistochemistry has taken longer than expected; and two, the technician who was to carry-out the bulk of this project developed tendonitis in both wrists. For nearly nine months she was on reduced service or out of the laboratory altogether due to wrist surgery. She rejoined the laboratory in August 2004 and is picking up the project again.

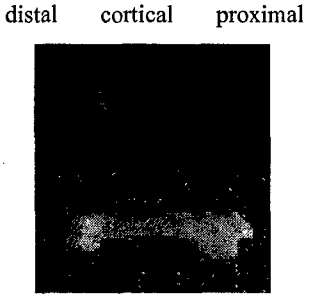

Figure 1. Fluorescent and brightfield images showing GFP-MDA-MB-435 cells in a femur from a nude mouse 4 weeks after intracardiac injection.

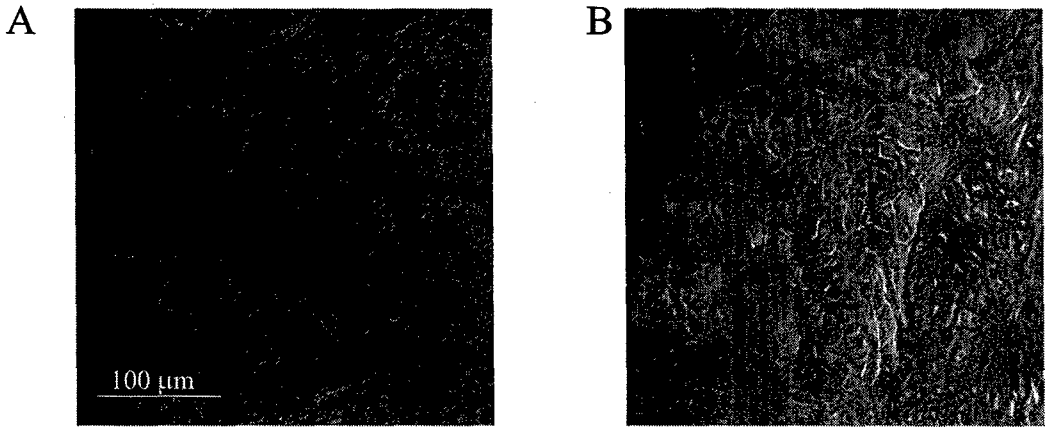

Figure 2a. Femurs were fixed, decalcified and cryosectioned. Three sections (approximately 20 microns) from different areas of the bone were examined by confocal microscopy. Approximately six to twelve fields were examined per distal and proximal regions and total GFP positive cells were counted. Very few cancer cells were seen in the diaphysis bone area.

Figure 2b. Femurs were fixed, decalcified, embedded in paraffin and sectioned (approximately 10 microns). Shown is an area of the distal metaphyseal region of the femur. GFP breast cancer cells are visible (green) surrounded by cortical bone.

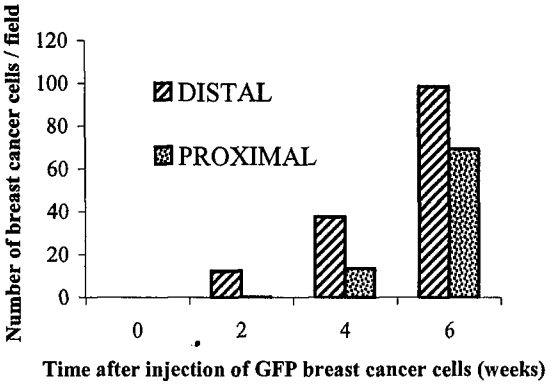

Figure 3. The experiment was carried out as described in the legend to Figure 2b. Shown are the average GFP breast cancer cells per field of bone.

Table 1. Flow cytometric analysis of GFP-MDA-MB-435 cells in the bone marrow of the femurs at various times after inoculation of the cells into nude mice.

Time Following Inoculation	Regions of the Femur			
	Proximal Metaphysis	Endosteal surface of the diaphysis	Central marrow of the diaphysis	Distal Metaphysis
1 hr	-	-	+++	-
4 hr	-	-	-	-
24 hr	-	-	-	-
72 hr	-	-	++	-
1 wk	-	-	-	-
4 wk	++	+/-	+	+++
6 wk	+	+	++	++

Athymic mice were inoculated into the left ventrical of the heart with GFP-MDA-MB-435 cells. At the times indicated, femurs were removed and cut into the distal and proximal metaphyses and the diaphysis. Bone marrow was flushed from these regions. The marrow in the center of the diaphysis was isolated separately from that close to the cortical bone. The red blood cells were lysed and the marrow cells were analyzed by flow cytometry using standardized fluorescent beads to estimate the total cancer cells present. Shown is a summary of the calculated values for the animals in each group.

(-) no GFP cells detected above background; (+/-) 10^2-10^3 cells; (+) 10^3-10^4 cells; (++) 10^4-10^5 cells; (+++) 10^5-10^6 cells.

KEY RESEARCH ACCOMPLISHMENTS

- Detection of GFP-breast cancer cells in distinct sections of the bone marrow cavity by flow cytometry and by immunohistochemistry and confocal microscopy.
- Detection of the shift in the position of GFP-breast cancer cells in the bone marrow cavity over time. First, the cells grew in the distal end of the bone. Only at the later stages (6 weeks) were significant numbers seen in the proximal region.

REPORTABLE OUTCOMES

- A senior undergraduate biotechnology major received a position in a new hospital based training program based partially on her experience in the laboratory helping with the histology part of the project.
- A junior undergraduate biochemistry and molecular biology major obtained a summer internship based partially on her experience in this laboratory helping with the histology part of the project.
- A small grant was applied for and awarded from the Pennsylvania Department of Health Breast Cancer Research Program based on the procedures developed in this project. This PDH award will allow us to assay for apoptotic osteoblasts in serial sections from the same bones.

CONCLUSIONS

At this point in the project, the conclusions are still tentative. However, it appears that breast cancer cells may first move in the bone to the distal metaphyses where they grow slowly or not at all before they move to the proximal regions. Once the cancer is well established the cancer cells are found in the proximal end. Seldom are they seen growing in the diaphysis. . By the time the project is complete we expect to determine if cancer growth precedes bone break-down. We also expect to obtain information on the cytokines, growth factors and chemoattractants released by bone and bone marrow.

So what? Currently metastatic breast cancer is treated primarily as a disease of the osteoclasts. Until we understand the role of the bone and the movement of the breast cancer cells in that milieu in the bone marrow there will be no effective cure.

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