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14. ABSTRACT

This study focuses on identifying differences in the vasculature in the ends of long bones where breast cancer cells tend to lodge, as compared to the vasculature of the central marrow cavity. We developed a method to isolate highly pure populations of both cell types. We found differences in mRNA using microarray analysis and confirmed the data by RT-PCR. The bone-derived cells express five messages in greater abundance P2-fold or more) than the marrow-derived cellsR conversely, the marrow-derived cells express higher levels of two other mRNAs. Possible roles may be fostering angiogenesis and cell survival. Using immunocytochemistry, we also found that the bone-derived cells present more of a cell surface adhesive protein, E-selectin. Taken together, the data show that the bone and marrow vasculatures are notably different in ways that could foster tumor growth within the bone compartment. At least one difference, surface presentation of E-selectin, is likely to be a factor in the specificity breast cancer cells have for bone environment.

15. SUBJECT TERMS
Cancer cells, Adhesive Proteins, Trabecular Bone, Vascular Endothelial Cells

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Introduction

The goal of this research was to identify characteristics of the cellular environment in the ends of long bones that may foster the entrapment of metastasized breast cancer cells. More specifically, we are focusing on the vasculature for two reasons. One, studies published by others indicate that the endothelial cells in iliac crest biopsies express more surface adhesive proteins than other vascular endothelial cells (1). Two, the vasculature in regions where breast cancer cells lodge are the first tissue barrier the cancer cells encounter. We have developed a method to isolate endothelial cells from the ends of long bones and are comparing the bone vascular endothelial cells (BVECs) with marrow-derived vascular endothelial cells (MVECs) using immunodetection of surface adhesion molecules and microarray analysis.

Body

Task 1. Compare endothelial cells of the microvasculature from the ends of long bones with their counterparts from the central marrow cavity. The first task was to isolate vascular endothelial cells from the ends of long bone, where the vascular cells are close to and aligned with trabecular bone surfaces; these cells are designated as BVECs. At the same time, vascular cells were isolated from the central marrow cavity; these cells are called MVECs. After testing several cell isolation methods, we chose a magnetic bead method as the best means to obtain cell populations which had high integrity and purity.

The magnetic bead cell isolation method was as follows. Eleven female Swiss Webster mice, 7-9 weeks of age were euthanized by CO_2 inhalation. Tibias and femurs were removed and stripped of extraneous muscle and connective tissue. Bones were split longitudinally, exposing the central marrow cavity and metaphyseal region. Marrow was removed from the central third of the bone shaft and placed in DMEM (Sigma, St. Louis, MO) culture medium; remaining marrow in the shaft and metaphysis was flushed out and discarded. Metaphyseal bone was then scraped with a No. 1 curette into DMEM to obtain trabecular bone fragments with adherent vascular endothelial cells.

The metaphyseal (BVEC) and marrow (MVEC) isolates were treated with 0.1% collagenase for 30 minutes at 37ºC. Cell preparations were centrifuged at 1200 rpm and resuspended in Medium 199 (MediaTech, Herndon, VA) supplemented with 20% fetal bovine serum (Sigma), 1% penicillin/streptomycin (MediaTech) and 1X endothelial cell growth factor (Sigma). Cells were plated in 60mm tissue culture dishes coated with 2% gelatin and incubated at 37ºC with 5% CO_2 humidified atmosphere. Cells were cultured for 7 days; media was changed every other day.

Endothelial cells were separated from other contaminating cell types using the Miltenyi VarioMACS magnetic cell sorting system (Auburn, CA). After one week, BVEC and MVEC cultures were harvested with the cell dispersion solution Accutase (Innovative Cell Technologies, San Diego, CA) and resuspended at a density not exceeding $5X10^6$ cells/500µl in a labeling buffer consisting of PBS with 2mM EDTA. The isolectin B4 from *Griffonia (Bandeirea) simplicifolia* seeds binds preferentially to mouse endothelial cells (2) and so was used to label the endothelial cells for magnetic sorting. The biotinylated isolectin B4 (Vector Laboratories, Burlingame, CA) was diluted 1:50 in the each cell suspension and incubated at 4ºC for 30 minutes. Cells were washed twice with labeling buffer then resuspended in 90µl of labeling buffer. Magnetic microbeads coated with streptavidin (Miltenyi) were added to the

labeled cell suspension and incubated at 4º for 15 minutes. Cells were washed twice with labeling buffer and resuspended in 500µl of separation buffer consisting of PBS with 2mM EDTA and 0.5% calf serum. A MACS separation column was prewashed with separation buffer and placed in the VarioMACS magnetic stand. A cell suspension was then loaded into the reservoir of each column and allowed to flow through the column. Non-endothelial cells that were not labeled with the biotinylated isolectin lacked streptavidin coated magnetic microbeads attached to their surfaces and thus would not be retained in the column. All columns were rinsed twice with separation buffer then removed from the magnetic field. Magnetically labeled endothelial cells retained in the column were then flushed out in 1 ml of separation buffer. Cells suspensions were centrifuged at 1200 rpm and resuspended in Medium 199 supplemented with 20% FBS, 1% penicillin/streptomycin and 1X endothelial cell growth factor (ECGF). Cells were plated at a density of approximately 50,000 cells/cm^2 in gelatin coated 35 mm plates and incubated at 37ºC for 7 days with media changes every other day. Cultures of putative endothelial cells isolated by this method were checked for purity by assessing their ability to take up fluorescent labeled acetylated LDL at an accelerated rate (3).

After one week in culture BVECs and MVECs were harvested. Total RNA was extracted from both cell types using the Qiagen RNeasy kit (Valencia, CA). Microarray labeling, hybridization and analysis was conducted on the RNA by the Penn State University DNA Microarray Facility under the direction of Dr. Craig Praul. Briefly, RNA obtained from three separate isolations of BVECs and MVECs was labeled using the Affymetrix GeneChip Expression 3' Amplification One-Cycle Target Labeling kit (Santa Clara, CA). After biotin labeling of the antisense strand, generated cRNAs were fragmented and hybridized to the Affymetrix GeneChip Mouse Genome 430A 2.0 array. Quality of the starting total RNA, the intact cRNA and the fragmented cRNA were assessed with the Agilent Bioanalyzer (Palo Alto, CA) and RNA 6000 Nano Lab chip. All RNAs at all stages were deemed of superior quality for analysis. Arrays were read by the Affymetrix GeneChip scanner and interpreted by GeneChip Operating software version 1.3.

Microarray data analysis was performed by Qing Zhang at the Penn State University Bioinformatics Consulting Center. Fold increase or decrease values were determined by comparing expression levels of BVECs to the expression levels of MVECs averaged from three isolations, as shown in the table below.

Affymetrix #	Gene name	BVEC fold change over MVEC
1418752_at	Aldehyde dehydrogenase 3A1 (ALDH3A1)	+2.8
1415935_at	SPARC-related calcium binding (SMOC-2)	+2.6
1417256_at	CCAAT enhancer binding protein C/EBP-β)	+2.1
1417256_at	Matrix metalloproteinase 13 (MMP-13)	+2.0
1425789_s_at	Annexin 8 (ANX8)	+2.0
1449193_at	CD5-like antigen (AIM, Spα)	-4.2
1448416_at	Matrix GLA protein (MGP)	-2.3

These data support our hypothesis that bone-derived vascular endothelial cells (BVECs) have unique properties when compared to control cells (MVECs). Interestingly, four of the proteins expressed in greater abundance by the BVECs are proteins that are involved in angiogenesis, SMOC-2, C/EBP-β, MMP-13 and ANX8.

To verify the different levels of expression observed on the microarrays, relative quantitative PCR was performed on RNA isolated from BVECs and MVECs cultured from three separate cell preparations. Total RNA was isolated from the cells using the Rneasy Mini kit (Qiagen) with on-column DNase treatment. One microgram of total RNA was reverse transcribed from random decamer primers using the RETROscript kit (Ambion, Austin, TX). One-twentieth of this reaction was then used in relative quantitative PCR reactions to determine the levels of message for each of the proteins of interest. Levels of amplified PCR product were normalized to 18S RNA using QuantumRNA 18S internal standard primers (Ambion). PCR reactions were optimized by varying the cycle number to determine the linear range of the amplification. RNA that had not been reverse transcribed was used as a negative control. PCR reactions for each of the proteins of interest were performed using the forward and reverse primers and cycle numbers listed in Table 1. Thermocycler parameters for all reactions were: 95^{o} C for 30 s, 58^{o} C for 30 s, and 72^{o} C for 1 min. PCR products were separated by electrophoresis on a 1.5% agarose gel in 1X tris borate EDTA buffer and stained with ethidium bromide. Gel documentation was performed by the Kodak Gel Logic 100 Imaging System (Eastman Kodak, Rochester, NY) and band volume quantitation was done by ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

As illustrated in figures 1 and 2, the RT-PCR quantitation of each mRNA verifies the findings of differential expression between bone-derived and marrow-derived vascular endothelial cells.

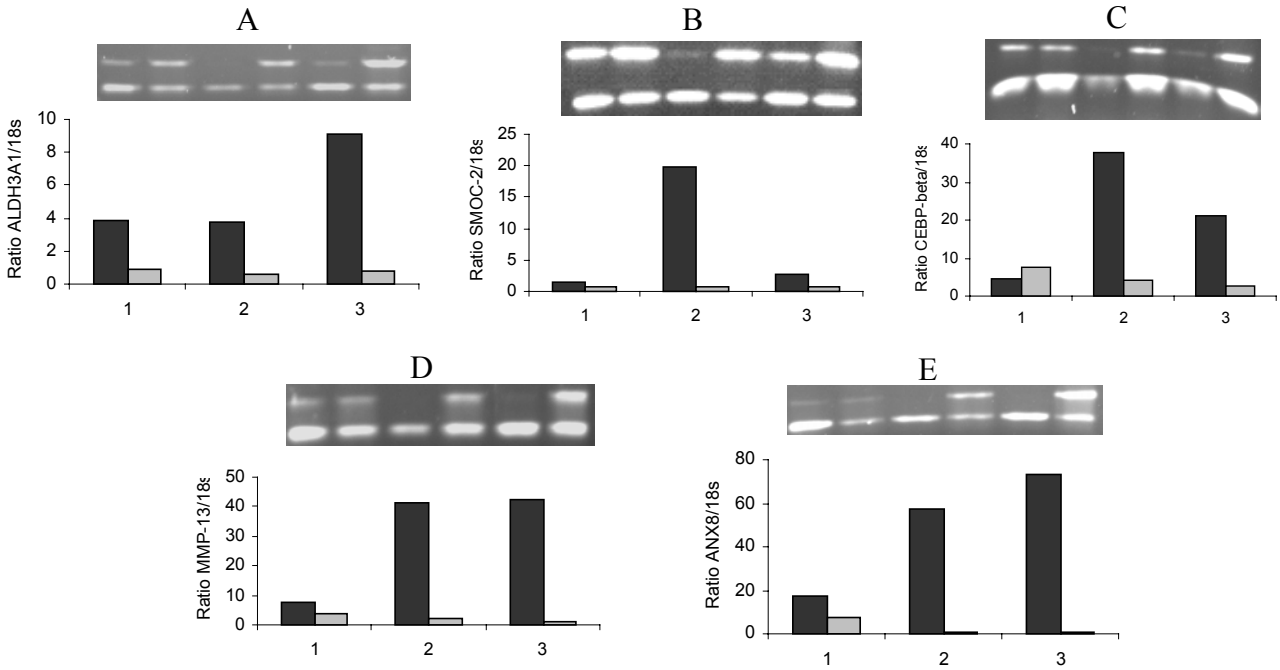

Figure 1. Relative quantitative PCR results for mRNAs that were expressed in greater abundance in BVEC than in MVEC. A) ALDH31A, B) SMOC-2, C) C/EBP-ß, D) MMP-13, E) ANX8. BVEC denoted by ■; MVEC denoted by ■ .

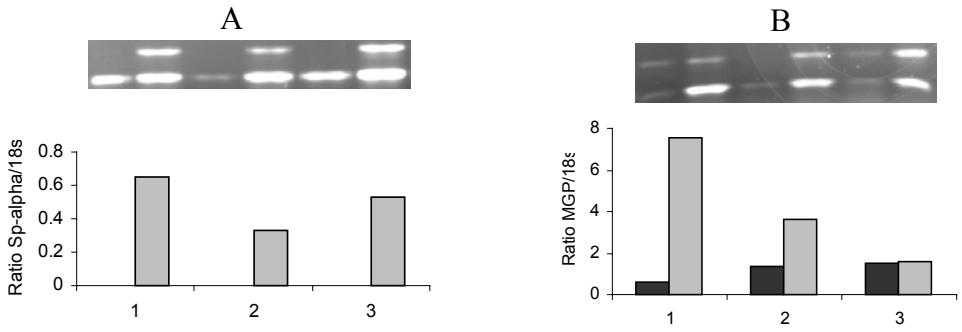

Figure 2. Relative quantitative PCR results for mRNAs that were expressed in greater abundance in MVEC than in BVEC. A) Spα, B) MGP. BVEC denoted by ■; MVEC denoted by ■ .

Task 2. Determine the difference in attraction of the breast cancer cells to the two types of vascular cells. Based on studies done by Lehr and Pienta (1), we postulated that the surface adhesion molecules P-selectin, E-selectin, ICAM and VCAM are expressed constitutively on the endothelial cells associated with trabecular bone and that these molecules could serve to tether metastasizing breast cancer cells to the endothelium and aid in extravasation. Initially, we expected to see that BVECs expressed these adhesion molecules at a higher frequency than MVECs. In our first experiments we isolated BVECs and MVECs as in task 1 and stained them for the presence of surface p-selectin, e-selectin, ICAM and VCAM. We found no significant differences in adhesion molecule expression between the two cell types (Figure 3).

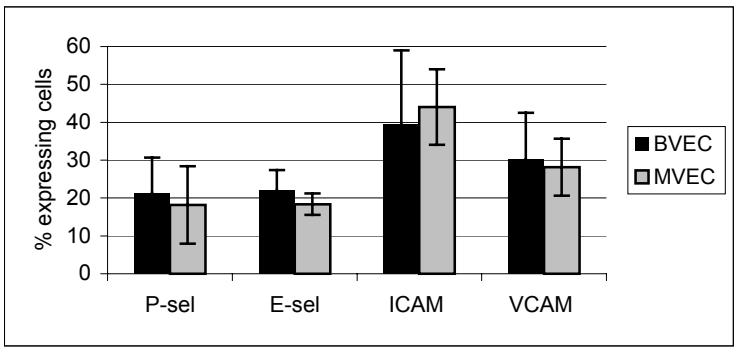

Figure 3. Basal levels of surface expression of tethering (p-selectin, e-selectin) and adhesive (ICAM, VCAM) proteins by BVECs and MVECs.

Because no differences were found, we then hypothesized that a secondary cell type, specifically osteoblasts, present in the bone microenvironment may be responsible for the upregulation of adhesion molecules on the surface of the adjacent endothelial cells. Osteoblasts lining trabecular bone in the metaphysis are numerous and are located in close proximity to the BVECs; MVECs in the central marrow cavity, on the other hand, are almost never found close to

osteoblasts. Osteoblasts are known to secrete such proteins as TGFβ and VEGF, which could influence the presentation of adhesion molecules on the surface of the endothelial cells (4,5).

Three separate isolations of MVECs and BVECs were cultured 7 days in Medium 199 supplemented with bovine serum and endothelial cell growth factor. The vascular cells were then exposed to conditioned medium derived from mouse osteoblast cell line (MC3T3-E1) for 24 hours. Since secretions of osteoblasts vary according to their stage of development, conditioned media from both immature (5-7 day culture) and mature (29-31 day culture) osteoblast cultures were used. To demonstrate adhesion molecules on the cell surfaces, BVECs and MVECs were exposed to antibodies against P-selectin, E-selectin, ICAM-1 and VCAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA). A fluorescent secondary antibody (Molecular Probes, Eugene, OR) was applied to intact, living cells. This ensured that only cell surface proteins would be localized. Then cells were thoroughly rinsed and fixed. The cells were visualized and counted using confocal microscopy. As shown in Figure 4, MVECs treated with medium from immature osteoblasts or medium from mature osteoblasts were not significantly different from each other or from the control treatment.

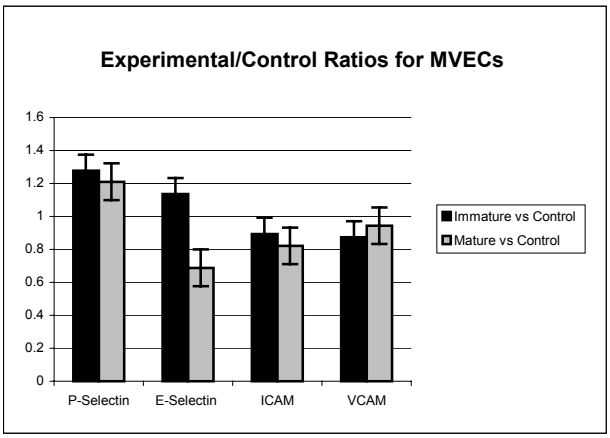

Figure 4. MVECs treated with osteoblast secretions. No significant responses were found.

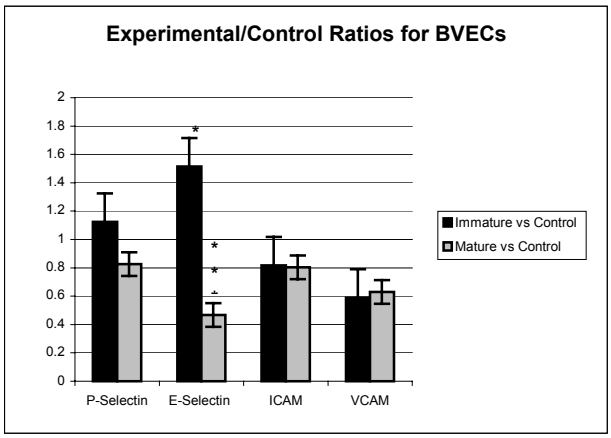

Figure 5. BVECs treated with osteoblast secretions. Surface expression of e-selectin was significantly altered.

BVECs responded in a differential manner (Figure 5). E-selectin was present on 33% more BVECs when treated with *immature* osteoblasts CM when compared to control media, E-selectin was lower by 53% when treated with *mature* osteoblast CM. The data suggest that *in vivo* endothelial cells juxtaposed to immature osteoblasts are upregulated for surface expression of e-selectin and may participate in tethering cancer cells in the blood stream at a higher frequency than other cells. This differential response of BVECs to osteoblast secretions supports our hypothesis that there are inherent differences between endothelial cells of the central marrow cavity and those of the metaphysis, a site of preferential metastasis for breast cancer cells.

Task 3. Test if proteins identified in Task 1 support breast cancer cell-endothelial cell interactions. This task was not attempted because Tasks 1 and 2 required more than the estimated time.

Task 4. Analyze, organize and publish the data. A paper on surface adhesion proteins has been published (Makuch *et al.*, 2006) and a paper on the microarray comparisons is in the final stages of completion.

Key Research Accomplishments

1. Improved the endothelial cell isolation method by employing magnetic sorting technology.
2. Obtained high quality RNA from BVECs and MVECs.
3. Found that secretions from immature osteoblasts stimulate BVECs to express surface e-selectin and that MVECs were unresponsive to both types of osteoblast secretions. This finding may help explain why breast cancer cells most frequently metastasize in bone.
4. Compared BVEC and MVEC RNA by microarray analysis and found 7 proteins differing by 2-fold or more.

Reportable Outcomes

Publications

1. Makuch, L. "Specificity of Breast Cancer Cells for Bone: The effects of osteoblast secretions on vascular endothelial cells," Honors Thesis, The Pennsylvania State University, 2005.
2. Gay, C.V., Makuch, L.A., Geffel, D.L. and Sosnoski, D.M., "Properties of well-vascularized regions of bones that become colonized by breast cancer cells," Era of Hope Conference, 2005 (abstract).
3. Gay, C.V., Makuch, L.A., Geffel, D.L. and Sosnoski, D.M., "Enhanced expression of E-selectin on bone-derived vascular endothelial cells: Potential role in breast cancer metastasis," Am. Soc. for Bone & Mineral Research Annual Meeting, 2005.
4. Makuch, L.A., Sosnoski, D.M. and Gay, C.V., "Osteoblast conditioned media influence the expression of E-selectin on bone-derived vascular endothelial cells," J. Cell. Biochem. 98:1221-1229, 2006.
5. Sosnoski, D.M. and Gay, C.V., "Evaluation of bone- and marrow-derived vascular endothelial cells by microarray analysis," in preparation.

Personnel

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Lauren Makuch

Conclusions

We have made and reported on two important discoveries during the course of this work. First, using RNA microanalysis and RT-PCR analysis, we have found five proteins that expressed in greater abundance in the bone-derived cells. These are: Aldehyde dehydrogenase 3A-1, SMOC-2, C/EBP-β, MMP-13, and Annexin 8. The role of ALD3A1 is likely to hydrolyze cytotoxic aldehydes in metabolically active bone. The role of SMOC-2 (Secreted Modular Calcium-Binding Protein-2) is probably involved in microvessel formation. C/EBP-β may act by upregulating IL-6, which is known to stimulate vascular cells to produce surface adhesive proteins. MMP-13 facilitates cell trafficking, including cancer cells, across blood vessel walls. Annexin 8 on cell surfaces is an anticoagulant; this has relevance since blood flow in trabecular bone regions is sluggish and therefore more prone to coagulation. Message for two proteins was greater in marrow-derived vessels. These are: SP_{α}, an apoptosis inhibitor, and Matrix gla protein, a protein that stimulates endothelial cell proliferation. Both SP_{α} and MGP may work together to protect and renew cells, where a great deal of intracellular trafficking (i.e. wear and tear) occurs.

The second important discovery was that the secretions of osteoblasts stimulate bone-derived vascular endothelial cells to present more E-selectin on their surfaces than control cells. This is significant because breast cancer cells are known to lodge in the ends of long bone and have surface ligands that bind to E-selectin.

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