The Role of Mechanical Rigidity in Cell Motility and the Progression of Cancer Metastases in Bone

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KEYWORDS. Cancer metastasis, Bone rigidity, Cell migration

BRIEF. A study on the behavior of breast cancer cells in response to bone's mechanical stimuli

ABSTRACT. Breast cancer regularly metastasizes to the bone microenvironment, where approximately 70 percent of all patients upon autopsy have bone metastases. Previous studies have linked the different rigidities of bone and breast tissue with the regulation of parathyroid hormone-related protein (PTHrP) and Gli2 expression, key factors involved in bone degradation. Upon exposure to a rigid surface like bone, breast cancer cells contract more frequently and have a greater expression of PTHrP and Gli2. In conjunction, we hypothesized that cell motility increases on mechanically rigid 2-D polyurethane scaffolds (PURS) and recently developed 3-D scaffolds. Multi-cell migration was investigated on rigid and soft scaffolds, and for targeting single cells, the cells per scaffold were initially optimized, and they were tracked over a set time interval. This project shows that tumor cells established in hard 2-D PURS demonstrate a higher migration rate than soft scaffold cells, and similar results were observed for 3-D PURS. Furthermore, the results from this study with ongoing experiments will likely show increased cell motility to be an important mechanism through which tumor-induced bone destruction is propagated.

INTRODUCTION.

Breast cancer is the second leading cause of cancer-related death in women, after lung cancer [2009, ACS Cancer Facts & Figures]. Lung and breast cancer are prone to metastasis, the movement of tumor cells to other organs in the body and establish secondary tumor sites. Bone is a common secondary site to which breast cancer regularly metastasizes [1]. Bone allows cancer cells to establish and grow, ultimately resulting in bone destruction causing pain and increased fracture risk in patients [2]. Also, patient survival is decreased by 71 percent for those who develop bone metastases compared to patients with localized breast cancer [1]. Current treatments for tumor-induced bone disease are limited, and focus on preventing bone destruction instead of inhibiting tumor growth in bone.

Bone destruction is propagated through the "vicious cycle of bone destruction", which disrupts the relationship between bone forming cells (osteoblasts) interacting with bone resorbing cells (osteoclasts) to remove older bone and replaces it with new bone [3,4]. However, upon tumor cell establishment in the bone microenvironment, growth factors present, such as transforming growth factor-beta (TGF-beta) stimulate the tumor cells to release bone resorbing factors, such as parathyroid hormone related protein (PTHrP), found to be regulated by a transcription factor, Gli2 [5,6]. PTHrP creates an imbalance between the osteoblasts and osteoclasts resulting in increased bone resorption [7]. TGF-beta is released from the bone matrix, promoting further tumor growth and PTHrP expression [5].

The physical properties of the skeleton have profound effects on cancer progression, and, more specifically, the vicious cycle of bone destruction. Previous research has reported a significant increase in PTHrP and Gli2 expression on rigid in vitro matrices in comparison to the expression on similar soft matrices [8]. Mechanical stress plays a role in tumor growth through the rigidity of the bone matrix in the secondary site [8]. When a force is applied on normal cells, an equal and opposite reaction occurs in the form of actin remodeling through a process called mechanoreciprocity or tensional homeostasis [9]. This stress leads to the activation of cell surface integrins, which stimulate Rho-kinase-dependent phosphorylation of MLC (myosin light-chain) kinase and subsequently, the interaction of myosin and actin [10]. Tumor cells have altered tensional homeostasis and generate more force than needed to counteract the original tension, thus further increasing actomyosin contractility [9].

This study measured the effects of differing scaffold rigidities on cell motility through whole cell population and individual cell tracking. Previous studies by other groups studying tissue rigidity used 2-dimensional polymer scaffolds as well as Matrigel as a template for in vitro bone research [8, 11]; however, all studies were conducted with low moduli scaffolds, but we used both 2- and 3-dimensional scaffolds at higher moduli as an indicator of cell motility. To compare overall cell migration on different surfaces, a scratch assay was used to measure the percent of the scratch remaining over a three day interval as an indicator of cell motility. We tracked individual cell motility over time using a Live Cell Imaging system, in which we monitored movement over time in cells growing on scaffolds with varying rigidities. These findings will help in understanding the effect of bone rigidity on tumor cell behavior.

MATERIALS AND METHODS.

Cell Culture.

The model for human breast cancer metastasis used was the MDA-MB-231(MDA) cell line. MDA cells were obtained from ATCC (American Type Culture Collection), and a bone metastatic variant was generated in our lab [2]. In addition, MDA-MB-231 cells were stably transfected with a plasmid expressing green fluorescent protein to create a MDA-GFP clone. MDA-MB-231 and MDA-GFP cells were incubated at 37° C with 5% CO2 in 1X DMEM (Dulbecco's Modified Eagle Medium) (Cellgro) supplemented with 10% heat inactivated FBS (Fetal Bovine Serum) (Atlas) and 1% Penicillin/ streptomycin. Cells were harvested by rinsing with PBS (Phosphate Buffered Saline) and with incubating in trypsin, an adhesion cleaving protein. During time lapse microscopy, the Live Cell imager was used to maintain a 5% CO2 concentration and 37° C temperature.

2-D and 3-D Material Synthesis.

Biocompatible polyurathane (PURS) substrates were synthesized and characterized as previously published [8]. Briefly, an appropriate amount of polyε-caprolactone-co-glycolide triol was mixed with an HDI prepolymer and COSCAT 83 catalyst (Vertellus) for 20 seconds in a Hauschild SpeedMixer™ vortex mixer (FlackTek, Inc). The resultant mixture was poured into the wells of a tissue culture plate and allowed to cure for 24h at 60°C. PUR substrates were prepared from the 3000-Da triol (Soft) and from the 300-Da triol (Rigid) to mimic rigidities of basement membrane and bone respectively. 3-D PURS were prepared as described in the above section and cast around the Polystyrene (PS) teflon molds (3D Biotek). The PS mold was then leached using an organic solvent, Dicloromethane. The resulting scaffolds are 100% interconnected, with no residual PS.

Scaffold Preparation.

In order for cells to attach to the scaffolds, scaffolds were first coated with adhesion protein. For these studies, fibronectin, an adhesion protein commonly found on bone and Type 1 collagen, another bone adhesion protein, were used. Fibronectin (1mg/mL, Life Technologies) , was diluted in PBS to a concentration of 4μg/mL, loaded into the scaffolds, and kept at 4˚C for 24 hours or at 37° C for 1 hour after exposure. The collagen gel (Millipore 3-D Collagen Cell Culture System) was prepared in a 4:1:0.25 ratio of the collagen solution, the 5x PBS or compatible medium, and the neutralization buffer respectively and immediately kept on ice.

Scaffold Seeding.

Cells were harvested as previously described and counted using a BioRad Cell Counter. MDA cells were diluted and resuspended in 30-50 µL of complete DMEM. A 30-gauge needle was used to inject the cells uniformly throughout the scaffold. When collagen was used, cells are resuspended in 30-50 µL of the collagen gel and injected into the scaffolds to polymerize. Scaffolds were incubated at 37° C for 1 hour after seeding, and then complete media was added.

Scratch Assay.

In order to compare migration rate between cells on rigid and soft surfaces, MDA-MB-231 cells were plated onto 2-dimensional rigid and soft scaffolds, which were marked with reference points for imaging. Once cells reached 80-90% confluency, plates were scratched with a pipette tip perpendicular from the plate and through the reference points. The cells were rinsed with PBS to detach any loose cells. Images were taken from 0 hours to 68 hours after the scratch. Images were analyzed for percent scratch remaining with respect to the starting wound area.

Migration Assay.

While the scratch assay showed overall migration, we were also interested in distance travelled by individual cells. MDA cells were also seeded on 2-D and 3-D rigid and soft surfaces. For the 2-dimensional studies, 2.5 x10^5 MDA-MB-231 cells were added to the scaffold wells. The plates were imaged using Live Cell time lapse microscopy once per hour for 24 hours. For the 3-dimensional studies, a 1:1 ratio of MDA-MB-231 cells and MDA-GFP cells were seeded in order to have fewer visible cells per image, but still have normal cell-cell interaction. Both cell lines were harvested and 10^5 cells total were injected into each scaffold. Images were taken over a 48 hour time period using GFP microscopy.

Image Analysis.

All image analysis was done using ImageJ (NIH) software. The scale was set in terms of micrometers per pixel. The area of the scratch in the scratch assay was measured and quantified using the freehand tool within the software. The percentage of scratch remaining was analyzed for all areas measured in terms of the original area for each condition. The multi-point tool was used for recording the coordinates of each cell in each picture. The distance travelled was calculated using the distance formula for each set of coordinates. Three cells per scaffold rigidity were tracked over time for total distance travelled.

Statistical Analysis.

All statistical analyses for experiments were performed using Microsoft Excel. Values are presented as mean ± standard error of the mean (SEM), and p-values determined using unpaired t-test, where *p< 0.05, **p< 0.01, ***p<0.001 unless otherwise stated.

RESULTS.

The method of injection for the 3-D scaffolds is very inefficient and varies the cell growth in each scaffold. Preliminarily, in order to optimize the loading of the cancer cells into the 3-D scaffolds, cells on fibronectin- and collagen-coated scaffolds were tested for proliferation. Cell retention was also tested by measuring GFP fluorescence after injection (Supplementary Figure 1). The scratch assay was a measure of the whole cell population's movement on rigid and soft matrices shown in Figure 1, while individual cells were tracked for distance traveled on 2-D and 3-D scaffolds shown in Figures 2 and 3 respectively.

Collagen coated scaffolds did not enhance cell proliferation.

Type-1 collagen was coated on the scaffolds and tested for cell proliferation compared to fibronectin coated scaffolds, fibronectin injected scaffolds, and uncoated scaffolds. Fibronectin was both pre-coated into the scaffolds and injected into the scaffolds with the cells. GFP fluorescence measurements were normalized to the control average, which were scaffolds in the absence of tumor cells. There was no significant difference between the GFP fluorescence measured of any two conditions on day two (Supplementary Figure 1). Each condition had extremely variant GFP measurements among its six replicates, which caused a high standard deviation. This is likely attributed, as previously stated, to the variant scaffold injecting procedure. On day one, scaffolds coated in type-1 collagen had a significantly higher amount of GFP fluorescence than each of the other three conditions, indicating that injecting cells within collagen more efficiently fills the scaffolds with cells.

Whole cell population migrates farther on rigid than on soft 2-D surfaces.

In order to compare migration rates between cells on rigid surfaces and soft surfaces, we performed a scratch assay on 2-dimensional scaffolds. Cells were seeded on both surface conditions. Over a 68 hour time interval, MDA cells closed the scratch about 6 times faster on rigid scaffolds in comparison to soft scaffolds, calculated in percent scratch remaining (Figure 1). Similar behavioral changes have previously been observed in PTHrP expression and invasiveness [9,13]. The control plate has rigidity similar to the hard scaffolds, but not as high, to mimic the bone. It is noteworthy that the cells reflect the same trend in the control as seen in the hard scaffolds.

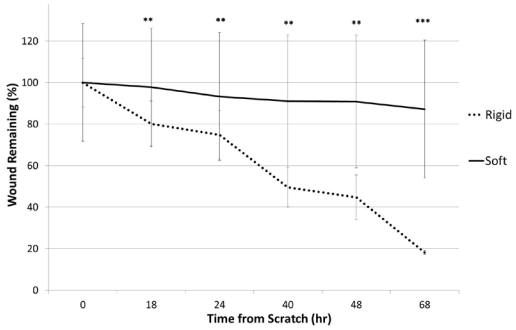

Figure 1. MDA-MB-231 cells were plated, grown to full confluency, and then split into two halves by a scratch to measure the amount of time it took to close the empty region by percent scratch remaining in each image taken. Rigid and soft scaffolds were compared. P-value, **= 0.01, ***= 0.001, measured between rigid and soft percentage.

Individual cancer cells travelled further on rigid 2-D and 3-D scaffolds.

Empty space is created in the scratch assay in order to drive tumor cell movement. On the other hand, cell movement was tested on individual cells without this empty space. MDA-MB-231 cells plated with optimal cell density on 2-dimensional scaffolds were individually tracked over a 20 hour time interval for distance travelled. Migration distance was almost 2 times greater on bone-like 2-dimensional scaffolds than on soft scaffolds (Figure 2). Moreover, greater rigidity increased individual cell motility without the influence of the whole population as in the scratch assay. Cells were seeded on rigid and soft 3-dimensional scaffolds and similarly imaged. Distances travelled were determined for each condition, and cells injected into rigid 3-dimensional scaffolds showed about six times the distance travelled with respect to soft 3-dimensional scaffolds (Figure 3). Together, these data and the scratch assay data support the hypothesis that an increase in matrix rigidity causes cell motility to increase and subsequently cell migration.

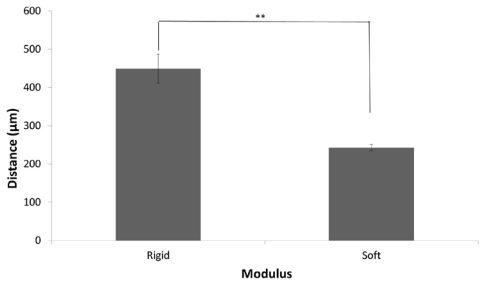

Figure 2. MDA-MB-231 cells were plated on 2-D scaffolds in order to track their individual distance travelled without influence of whole-population force such as in the scratch assay. Three individual cells per rigidity were tracked for total distance travelled. P-value **= 0.01.

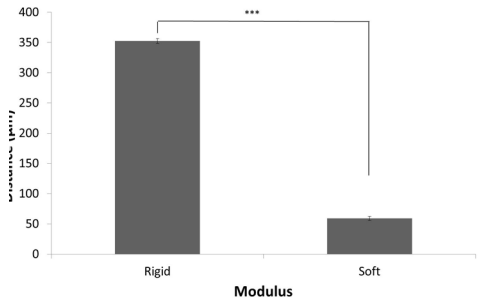

Figure 3. Similar to the 2-D scaffolds, 3-D scaffolds were injected with MDA-MB-231 cells to track their individual distance travelled without influence of whole-population force such as in the scratch assay. Three individual cells per rigidity were tracked for total distance travelled. P-value ***= 0.001.

DISCUSSION.

Metastasis of breast cancer to the bone environment substantially decreases patient survival and exhibits an overall greater malignancy compared to localized breast cancer [2]. It was hypothesized that the higher stiffness of bone compared to soft tissue increases cell motility and migration, which contribute to tumor malignancy through the spread on bone. The mechanical rigidity of bone has already been found to induce changes to tumor cell behavior, shown in past research. Bone rigidity is a factor that has been found to regulate PTHrP and Gli2 expression and has even proven necessary for TGF-β stimulation of PTHrP and Gli2 expression [8] Studies showed that higher rigidity caused a greater malignant phenotype [12] and invasiveness [13] due to Rho-GTPase-dependent actomyosin contractility [9].

It has become increasingly evident that bone rigidity has a profound effect on tumor cell behavior in respective metastases. The cell's ability to sense rigidity through mechanotransduction is an important factor in the initiation and propagation of breast cancer metastases into bone tissue [8]. Much of these behavioral changes observed in tumor cells stem from their altered tensional homeostasis or the abnormal increase in cytoskeletal forces as a result of extracellular stress [9]. Inhibition of ROCK mechanotransduction signaling has been found to decrease cell contractility and spread [9].

Although 3-dimensional scaffolds mimic bone better, the method of cell injection is not fully optimized, which can be supported by the smaller distances travelled by cells seeded onto 3-D scaffolds in comparison to 2-D scaffolds. Additionally for this project, fibronectin and type-1 collagen, a polymerizing gel, were tested for cell proliferation optimization. Despite no significant difference between cell proliferation among the conditions, scaffolds coated with type-1 collagen retained a greater amount of cells than each of the other conditions (Supplementary Figure 1), efficiently improving the method of injection.

On 2-dimensional scaffolds, the scratch assay indicated that cells closed the wound substantially faster on rigid surfaces compared to soft surfaces (Figure 1). Individually, cancer cells seeded onto rigid 2-dimensional surfaces also migrated a significantly larger distance in comparison to those seeded on soft surfaces. The difference in migration between hard and soft scaffolds was even more significant on 3-dimensional scaffolds (Figure 3). This correlates with preliminary data demonstrating that 3-dimensional scaffolds had greater expression of PTHrP and Gli2 than 2-dimensional scaffolds. Similar changes in tumor cell motility were observed in human prostate carcinoma cells (DU-145) [11], single population cells (SPC) derived from MDA-MB-231 cells [14], and smooth muscle cells (SMC) [15]. Migration also decreased on rigid surfaces when exposed to ROCK inhibitors [15], correlating with the decrease in actomyosin contractility [8]. Moreover, we speculate that the increased cell motility from our scratch and single cell tracking assays may be due to ROCK signaling and subsequently increased cytoskeletal forces.

CONCLUSIONS AND FUTURE DIRECTIONS.

Migration was measured in response to different mechanical stimuli, and the stiffness of bone has an increasing effect on migration of cells as a population, shown through the ability of the breast cancer cells to close a scratch wound, and individually, demonstrated through the distance tracking assay. In addition, collagen was tested against fibronectin as a better adhesion protein for cell injection into 3-D scaffolds and was found to increase cell survival during injection in comparison to fibronectin, the protein regularly used to coat the in vitro matrices. Gene expression experiments will be performed to compare the PTHrP and Gli2 expression of cells injected into scaffolds with the different coating proteins to determine which protein better models the tumor microenvironment in bone tissue.

Given that higher rigidity increases the cells ability to contract [9], this research is consistent with the idea that stiffness of bone increases cell migration through cell contractility. Mechanical rigidity plays an important role by means of osteolytic gene expression, and this project provides strong evidence that rigid surfaces increase tumor cell migration; however, no correlation has been investigated between increased cell migration and osteolytic gene expression or bone destruction. Our future goal is to link cell migration and the spread of tumor cells across bone to the detrimental effects of the vicious cycle of bone destruction. If a correlation can be made, cell motility and migration would be a viable therapeutic target for limiting bone fracture and destruction.

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SUPPORTING INFORMATION.

Figure S1. MDA-GFP cells were injected into 3-D scaffolds by the use of four different methods: Injecting cells on an uncoated scaffold, coating scaffolds in fibronectin before injecting cells, injecting fibronectin with cells, and injecting collagen with cells. GFP fluorescence was measured over a two day time interval. P-value *= 0.05, **= 0.01, measured to the collagen mixture day 1 (nothing else significant).

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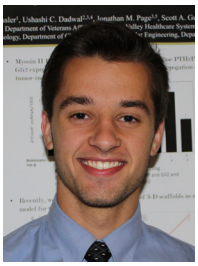

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