Award Number:
W81XWH-12-1-0172

TITLE:
Effect of Physical Forces on the Metastatic Bone Microenvironment

PRINCIPAL INVESTIGATOR:
Joseph L. Sottnik

CONTRACTING ORGANIZATION:
University of Michigan
Ann Arbor, MI 48109

REPORT DATE:
October 2013

TYPE OF REPORT:
Annual Summary

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
The overall goal of this project is to determine if prostate cancer (PCa) bone metastases increase intramedullary pressure (ImP). The central hypothesis to be tested is that tumors promote increased pressure thereby creating a more pro-tumorigenic environment. We have tested this hypothesis in vivo observing that tumors with an osteoblastic component do indeed lead to increased ImP. We have then utilized the pathologic pressures determined in vivo to perform in vitro experiments on PCa and bone cell lines. In vitro experiments have shown that increased pressure leads to decreased PCa proliferation. Osteoblasts also have inhibited proliferation, yet osteocytes have increased proliferation. Furthermore, conditioned media from osteocytes can promote an aggressive tumor phenotype when applied to tumor cells. Novel candidate genes with altered expression due to pressure have been identified and are currently undergoing further validation prior to functional testing. These results provide evidence that tumor induced pressure leads to a pro-tumorigenic environment and is the center of ongoing studies.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Body</td>
<td>1</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>4</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>4</td>
</tr>
<tr>
<td>Conclusion</td>
<td>4</td>
</tr>
<tr>
<td>References</td>
<td>5</td>
</tr>
<tr>
<td>Appendices</td>
<td>5</td>
</tr>
</tbody>
</table>
INTRODUCTION

Metastatic progression of prostate cancer (PCa) to bone is a clinical significant event with a high incidence and limited treatment options. Even though a significant amount of resources have been utilized to investigate the biochemical relationships in bone metastases, little has been done to investigate the physical forces created by tumor cells growing in the relatively non-plastic bone environment. The overall goal of this project is to understand the changes in physical forces associated with tumor growth. The primary hypothesis to be tested is that PCa bone metastases promote pressure increases in bone leading to pro-tumorigenic alterations in the microenvironment. This research is being undertaken as a postdoctoral fellowship under the mentorship of Dr. Evan Keller at the University of Michigan. We have made some initial progress concerning the measurement of physical forces in vivo and have begun to understand the mechanisms by which physical forces create a pro-tumorigenic environment utilizing in vitro assays.

BODY

Throughout the course of study and investigation, I, Joseph L. Sottnik, have been involved in a well-rounded training program at the University of Michigan. This training program includes participation in various seminars, and development of numerous collaborations within and outside the University of Michigan. I have been able to take classes in grant writing during this time. There has been significant basic, clinical, and translational exposure throughout the course of the program thus far. I have also had the privilege to attend national scientific meetings such as the American Association of Cancer Researchers Focus meeting on the tumor microenvironment and the American society of bone and mineral research annual meeting. I have also been able to attend local meetings present at the University of Michigan. Throughout the training program I have been able to make progress on the proposed project and have begun to illicit plausible mechanisms by which increased intramedullary tumor pressure can promote pro-tumorigenic changes.

Task 1.1 – Identification of pressure changes due to tumor growth

We have been able to implant mice successfully with the wireless transmitter and have found it necessary to implant tumors 24 hours prior to implantation of the transmitter. Large fluctuations in intramedullary pressure (ImP) are observed for the first 5-7 days after implantation which is likely due to inflammation. Tumor challenge with PC3 led to no change in ImP. PC3 is a very lytic tumor and we propose that the bone lysis associated with this tumor was able to dissipate the pressure, thereby leading to no alteration in ImP during tumor growth. PC3 cells were luciferase transfected, and measurements taken utilizing a Xenogen system did not lead to a significant (p>0.05) correlation between pressure and tumor growth.

Subsequent experiments were performed with ACE-1, a canine prostatic carcinoma cell line that is slower growing than PC3 but results in mixed osteoblastic/osteolytic lesions. We observed a significant increase in ImP 18 days after tumor challenge which then dissipated over time (Figure 1). Pressure decrease was associated with radiographic lysis of cortical bone. We hypothesize that the pressure dissipation was due to the lytic component of the disease and breach of the cortical bone by the tumor helping to relieve pressure from the lesion.

Due to the lack of information obtained from PC3, we sought to utilize DU145. DU145 is a slower growing tumor with radiographic lesions more similar to that of ACE1 than PC3. Again, we observed a steady increase in ImP, reaching a maximum pressure on day 16, and then a subsequent decrease in pressure (Figure 1). Again, this decrease in pressure was associated with tumor breaching cortical bone as measured by radiography.
Task 1.2 – Defining the impact of intramedullary pressure on tumor growth

Due to delays in determining the ability of tumors to increase ImP, we have yet to initiate studies involving manipulation of pressure through catheter implantation. We have determined that extraction of fluid will not be suitable for decreasing ImP. Therefore, we are investigating other methods such as hind-limb suspension and utilizing Botox injections to inhibit ImP.

Task 2.1 – Characterization of cellular viability and proliferation in response to hydrostatic pressure

Initial experiments utilizing the in vivo models in task 1 identified 10 and 30 mmHg as reasonable control and peak pressures due to tumor induced increases in ImP. These pressures were used for a number of the early experiments. After completion of the studies in Task 1.1, it was determined that across all tumor models, the average basal ImP was 19.12 mmHg and the peak ImP induced by tumor was 38.51 mmHg. Therefore, later experiments have employed 0 (un-pressurized), 20, and 40 mmHg for experimental conditions in the modified opticell system previously proposed [1, 2].

Proliferation as assessed by cell counting has been found to be the most reliable method for assessing proliferation. MC3T3 and ST2 have decreased cell numbers after pressure application for 24 hours (Figure 2). Conversely, MLO-Y4 have increased cell numbers after pressure application (Figure 2). Apoptosis has been assessed across all cell lines using Annexin

Figure 1: Tumor growth leads to increased ImP in mice. Mice were challenged with A) ACE1 or B) DU145 prostate cancer cells 24 hours prior to implantation of a wireless transmitter. Mice without tumor serve as controls. Mice were euthanized due to primary tumor size. Increased ImP is observed with both tumors.

Figure 2: Viability of cell lines treated with hydrostatic pressure. Cells were pressurized utilizing an in vitro hydrostatic pressure system previously described. Cells were treated with hydrostatic pressure for 24 hours prior to counting. A) LNCaP, B) MC3T3, and ST2 have decreased proliferation whereas MLO-Y4 increased proliferation.
V/FITC staining and flow cytometry. No significant change in apoptosis has been observed in any cell line. Therefore we can conclude that changes in cell number are primarily due to altered proliferation.

We have observed no significant alterations in apoptosis when tumor cell lines, PC3, LNCaP, DU145, or ACE1 have pressure applied (Figure 2). However, we do observe decreased cell numbers from all cell lines. Therefore, as above, PCa proliferation is inhibited at elevated pressures known to be induced by tumor in mice.

To better understand the pro-tumorigenic alterations associated with tumor induced increases in ImP, we have pressurized osteocytes and collected conditioned media (CM) from these cells. This scenario recapitulates tumor induced pressure on the bone microenvironment. We have observed that CM from pressurized osteocytes leads to increased proliferation, migration, and invasion of tumor cells. Experimental results with PC3 are shown as an example in Figure 3. Similar results have been obtained with PC3 and DU145. Osteocytes have not been previously implicated as pro-tumorigenic. The application of pressure appears to make osteocytes more tumorigenic. These are novel observations and ongoing experiments are being performed to understand the mechanism of action responsible for these observations. The implication of osteocytes may lead to novel pathways that can be targeted for therapeutic development.

**Figure 3:** Conditioned media from pressurized osteocytes promotes an aggressive cancer phenotype. Conditioned media (CM) was prepared with osteocytes under pressure. CM was then applied to PC3 tumor cells to assess A) proliferation. Modified Boyden-chamber assays were performed with CM in the bottom well to act as a chemoattractant. Increased B) migration and C) invasion of PC3 cells was observed with increased pressurization. Bars show significant differences compared to control as determined by one way ANOVA and significant cutoff of p<0.05.

**Task 2.2 – Identification of novel pathway activation due to hydrostatic pressure**

We have focused primarily on the factors induced by pressure on bone components, primarily osteoblasts and osteocytes. We have been able to pressurize cells, isolate RNA, and perform transcriptome arrays. However, the data obtained from these experiments was not sufficient for analysis. Targeted analysis of important genes from the literature were profiled using qPCR. However, few genes were found to be significantly altered in the presence of hydrostatic pressure. This may be due to many experiments being performed utilizing fluid flow which may illicit an independent response from that of hydrostatic pressure. *In vivo* tumor pressure is a mix of static and dynamic forces, as determined by our *in vivo* experiments, and both forces may be equally applicable. Therefore we continued by investigating alterations in Wnt and BMP signaling using commercially available targeted arrays as proposed. We observed alterations in the gene expression of Frat1, Rhoa, Gdf10, Dlx5, and BMP7 (Table 1). These alterations in gene expression may be mediators of pro-tumorigenic changes
due to increased ImP. Validation of these results by western blot is ongoing prior to functional evaluation of these targets.

Due to significant problems identifying alterations in novel genes, and those expected to be regulated by pressure, we have been unable to identify candidate genes for knock-down or over expression. These studies continue and will hopefully yield results shortly.

Task 2.3 – Validation of candidate genes in tumor bearing mice

Due to the difficulty in determining novel pathways and identifying reliable candidate genes of interested we have been unable to start probing tumor samples for alterations in any genes. Once greater progress is made in Tasks 2.1 and 2.2 we will be able to start investigating tumor tissue for these changes.

**KEY RESEARCH ACCOMPLISHMENTS**

- Determined that tumor growth increases ImP in mice
- Identified that cell viability is cell type dependent and primarily due to alterations in proliferation and not apoptosis
- Pressurization of osteocytes leads promotes an aggressive tumor phenotype
- Identified candidate genes responsible for a pressure phenotype

**REPORTABLE OUTCOMES**

None

**CONCLUSION**

We have observed that tumor bone metastases increase ImP. Pressures observed from in vivo experiments are acting as the foundation for ongoing in vitro experiments utilizing bone and tumor cell lines. We have observed that increased pressure leads to decreased proliferation of tumor cell lines. We have determined that pressurized osteocytes promote proliferation, migration, and invasion; a novel phenotype. We have identified candidate genes that may play a role in promoting tumor growth in the presence of increased pressure. Even though some of our in vitro experiments have been delayed due to difficulties in screening, we are progressing on functional studies to assess the importance of these candidates. Identification of novel mediators of bone tumor metastasis may be important in targeting new pathways.

<table>
<thead>
<tr>
<th>Gene</th>
<th>20 mmHg Fold Change</th>
<th>40 mmHg Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmp7</td>
<td>2.6635</td>
<td>1.9274</td>
</tr>
<tr>
<td>Dlx5</td>
<td>2.4737</td>
<td>1.7171</td>
</tr>
<tr>
<td>Gdf10</td>
<td>-3.2415</td>
<td>-1.154</td>
</tr>
<tr>
<td>Frat1</td>
<td>1.6591</td>
<td>2.3459</td>
</tr>
<tr>
<td>Rhoa</td>
<td>-1.079</td>
<td>-1.1592</td>
</tr>
</tbody>
</table>

**Table 1**: Gene expression changes from targeted microarrays of MLO-Y4 cells treated with pressure. MLO-Y4 cells were treated with 0, 20, or 40 mmHg for 24 hours before RNA isolation and targeted qPCR based array. Fold changes are relative to control (0 mmHg). Identified genes are candidates for further validation as important mediators of tumor induced pressure.
REFERENCES


APPENDICES

None