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14. ABSTRACT
Purpose: Understanding the influence of the dura and its secreted cytokines is the goal of the proposed project. Scope: We intend to examine the effects of dura and its released cytokines on the invasion and growth of prostate cancer cells in-vitro and in-vivo and explore a possible mechanism for these effects. Major findings: Over this period, we have found that when dura is exposed to tumor that it changes the dura and leads to increase in certain cytokines these do not significantly effect tumor growth, migration or invasion. It leads to increase in cytokine release of il-6 and other inflammatory cytokines

15. SUBJECT TERMS
Bone Metastases, Prostate Cancer, CXCR2, Dura

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Introduction

Cancer can spread to nearly all tissues of the body, one of the most common sites of spread is to the bones. The majority of these metastases are found in the spine. About two-thirds of all symptomatic bone metastases are located in the spine with up to 74% of patients having spine metastases at autopsy and a majority of these in the posterior part of the vertebral body. A key feature of the spinal skeleton local environment is the proximity of the dura mater adjacent to the posterior aspect of the vertebral body, the covering of the central nervous system. The immediate objective is to examine the dura and its effects on prostate cancer cells as well as the effect of tumor cells on the dura. Tumor cells change the environment to help themselves grow we examine this feedback loop and ways of blocking it. Because the dura is next to the vertebral body we look to molecules that the dura releases. These molecules act as keys that unlock doors to pathways that increase tumor malignancy and growth. We will use different methods to quantify the effect and determine the clinical significance of these keys in animal models.

keywords Bone Metastases, Prostate Cancer, CXCR2, Dura, Cytokines, CXCL1, CXCL8,

Accomplishments What were the major goals of the project? Examine the in-vitro effects of dural secreted factors on prostate cancer growth, survival, and invasion Examine the in-vivo effects of dura on prostate cancer initial invasion using animal models of spine metastases.

Specific Aim 1: Determine the effects of dura in the initial invasion and early growth of PCa spine metastasis

Major Task 1 (Aim 1.1): Examine the in vitro effects of duralsecreted factors on prostate cancer growth, survival and invasion.

We have done numerous experiments that show that the dural cytokines activate the CXCR2 receptor in prostate cancer and lead to increase growth and migration and that this effect is mitigated when a CXCR2 blocker is administered. We have finished all the in vitro studies . We observed high levels of cytokines produced by dura and within the vertebral body bone marrow, namely CXCL1 and CXCL2, that act on the CXCR2 receptor. All prostate cell lines treated with DCM demonstrated significant increase in growth, migration and invasion regardless of androgen sensitivity, except PC3, which did not significantly increase in invasiveness. When treated with SB225002, the growth response to DCM by cells expressing the highest levels of CXCR2 as measured by FACS (LNCaP and 22Rv1) was blunted. The increase in migration was significantly decreased in all lines in the presence of SB225002. Interestingly, the invasion increase seen with DCM was unchanged when these cells were treated with the CXCR2 inhibitor, except PC3 did demonstrate a significant decrease in invasion. These findings were published (Strong, M.J., Rocco, S., Taichman, R. *et al.* Dura promotes metastatic potential in prostate cancer through the CXCR2 pathway. *J Neurooncol* **153**, 33–42 (2021). https://doi.org/10.1007/s11060-021-03752-4)

Major Task 2 (Aim 1.2) : Examine the in vivo effects of dura on prostate cancer initial invasion using animal models of spine metastasis. In this model we proposed putting fragments of vertebral bodies in a subcutaneous compartment of a mouse and examining the effects of dura and no dura on tumor spread after a cardiac injection of PC3 cells. We have done numerous preliminary experiments with this model and found it to be wanting. Although this model had been utilized in the past and published on by our colleagues it is not very robust. We got only one mouse out of every 20 that developed tumor in the subcutaneous bone fragments. This is not enough to show anything statistically or to power a study. We applied for and made alterations to our animal protocol and model and will now inject tumor cells into the bone fragment before putting it subcutaneous. We will than use IVIS luciferase expression to follow the growth of these tumors in this model in a group of animals with CXCR2 inhibitor and one group without. This will show whether in vertebral bodies this inhibitor will modify the tumor growth and this could lead to human clinical trials using such an inhibitor See data below this was than used to examine tumor expansion not tumor initiation.

Major Task 3 (Aim 1.3): Examine the cytokine profile of human dura and the effect of DCM from human dura on PCa cells and surgically obtained human spinal metastatic cells.

We did examine human dura and examine its mrna from specimens showing similar cytokine profile to mouse. We attempted to culture human dura from patients and did get some fibroblast growth grow but never enough to creat the dural conditioned media with the volumes that were needed to perfom experiments on many cell lines. We did do cytokine profiling of the vertebral body and compared this to the cytokine profile of the long bones in mice and humans to examine for the presence of dural released cytokines in the bone marrow. We found dural cytokines in much higher concentration in the vertebral body bone marrow compared to the long bone. The work was published (Ahmed AA, Strong MJ, Zhou X, Robinson T, Rocco S, Siegel GW, Clines GA, Moore BB, Keller ET, Szerlip NJ. Differential immune landscapes in appendicular versus axial skeleton. PLoS One. 2022 Apr 27;17(4):e0267642. doi: 10.1371/journal.pone.0267642. PMID: 35476843; PMCID: PMC9045623.)

Specific Aim 2. Determine the effects of prostate cancer to direct the secretion of dural factors that drive further growth of metastases during the late stages of the disease

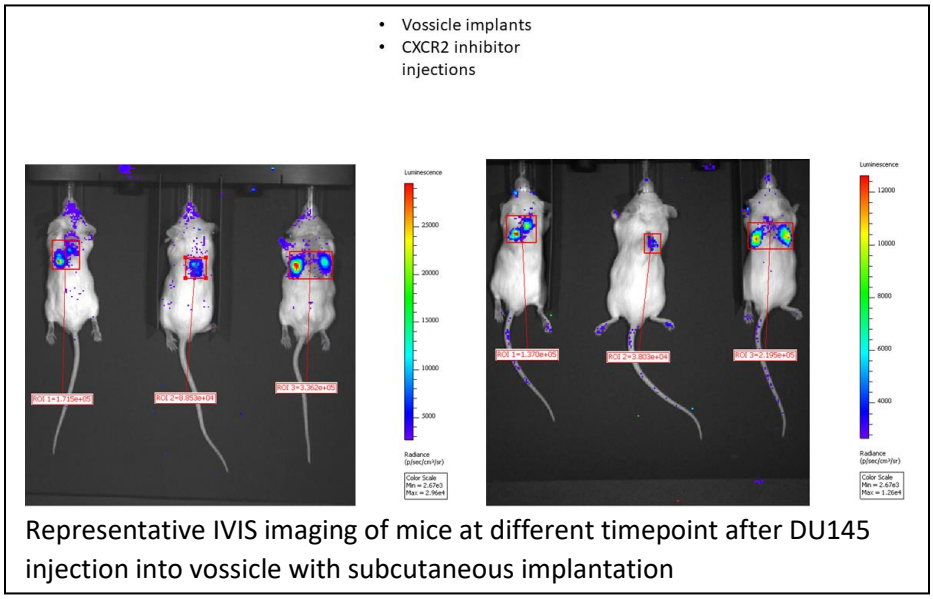
Major Task 1 (Aim 2.1) Validate the effects of cancer activated dural cells on PCa cell lines and patient derived spinal metastatic cells in vitro.

Our preliminary data suggested that CXCR ligand RNA expression is high in dura and increases when dura is exposed to tumor cytokines. This raised the idea of a reciprocal loop of increased expression of CXCR-ligand and increase tumor effects over time. We created dural conditioned media by growing mouse dura in culture and than grew it in non contact cocultures with tumor cells (PC3). We completed experiments looking at this conditioned media compared to the dura conditioned media. We examined growth, invasion, migration as we did previously(Major task 1 of aim 2.1). This could be a ceiling that is reached. We have done experiments now in all of cell lines. We saw that the dura conditioned media again increases growth and migration compared to plain media (with serum) but that the addition of the tumor conditioned dura media has little effect on growth or migration. Again the effect seen is mitigated by the CXCR2 inhibitor. This is despite the fact that the RNA

levels of the CXCR2 ligand increased on the RNA sequencing we originally observed. To further investigate this we performed ELISA measurements on the conditioned media and the tumor media to examine the cytokine levels in the media. (subtask 2 Aim 2.1b) described above. When this was done we found that the actual values of the ligand does not increase in the media even though we previously have seen RNA increases.

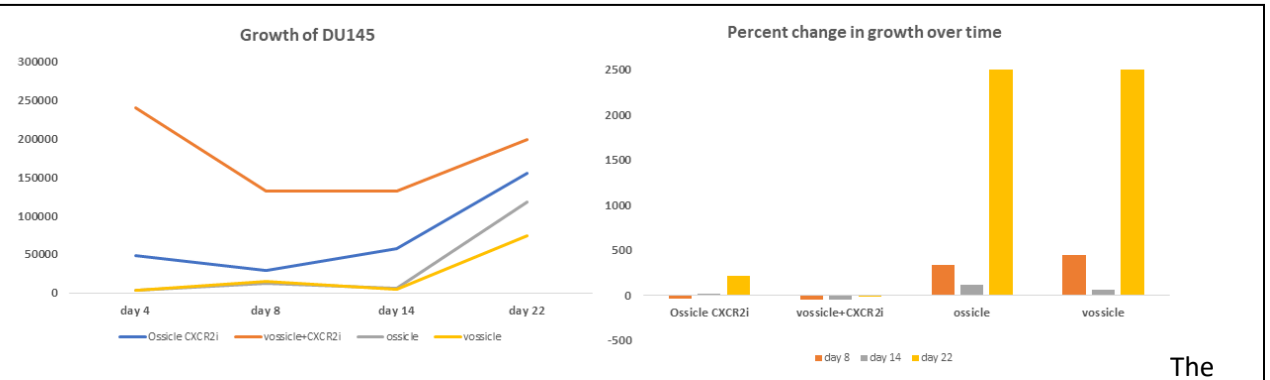
Major Task 2 (Aim 2.2) Examine the effects of cancer activated dura cells to intensify the expansion of prostate cancer bone metastasis in vivo.

We continue to make progress albeit slowly in this covid arena. We are examining the role of CXCR2 receptor and activation of this receptor in prostate cancer by dural cytokines For the SOW this is major task 2 subtask 1 this is the major experiment remaining to be completed



Representative IVIS imaging of mice at different timepoint after DU145 injection into vossicle with subcutaneous implantation

We have also completed our in-vivo experiments that I previously described examining the different rate of growth in a CXCR2 abundant cell line (DU145) in both ossicles and vossicle to examine differences between the different bone types vossicle (pieces of vertebral bodies) taken from a SCID mouse and ossicles (pieces of long bone) taken from SCID mice. We saw



The CXCR2i stops the growth of implanted Du145 cells in both bones compared to the group without inhibitor. In the Ossicle group there seems to be some late growth that is not seen in the Vossicle group with inhibitor.

significant increase in percent tumor growth in the condition without any inhibitor over 22 days of growth with about the same rate of growth between ossicles and vossicles. With the addition of the CXCR2 inhibitor we saw significant decrease in the rate of growth with actually initial decrease in growth after a few days of inhibition followed by a resumption of growth in the long bone group but not the vossicle group indicating

that CXCR2 expression in the tumor definitely seems important to tumor growth in the bones and there may be something about the vertebral body micro-environment that makes it even more reliant on the CXCR2 pathway. This was further investigated in numerous experiments examining differences in bone microenvironment of long bones and vertebral bodies (Ahmed AA, Strong MJ, Zhou X, Robinson T, Rocco S, Siegel GW, Clines GA, Moore BB, Keller ET, Szerlip NJ. Differential immune landscapes in appendicular versus axial skeleton. PLoS One. 2022 Apr 27;17(4):e0267642. doi: 10.1371/journal.pone.0267642. PMID: 35476843; PMCID: PMC9045623.)

Overall we completed many of the tasks related to our major tasks and specific aims. I we have published two papers related to this work and answered the overall question of the importance of CXCR2 in prostate cancer. We did abandoned some of the smaller experiments that either did not pan out scientifically or fit with the data we were getting (we abandoned CXCR4 inhibition due to a lack of significant results) We attempted to examine the amount of CXCR2 in prostate cancer specimens and could not get a good quantifiable staining protocol in anything but cells.(it did not work in processed specimens or bone). From here we will be examining the relationship of cancer to the specific tumor microenvironement not only does dura release these cytokines that have an effect on the tumor we see very different cell populations in the vertebral body compared to bone without dura indicating an effect on the local microenvironment that may play a role in tumor initiation and growth.

Impact: We think that the results we have seen so far would warrant moving forward with a clinical trial in the future with CXCR2 inhibitors. This research begs further research to examine the effects of CXCR2 in an immune competent system as well as the impact of CXCR2 on the local microenvironment which we will begin investigating in future grant mechanism. It has also led us to examine the immune micro-environment much closer and we are currently going through that data which shows clear differences in immune cell populations between long bones and vertebral bodies..

Changes/Problems There have been changes (see above) we had to abandon the intracardiac injection model after a few preliminary attempts had such a low rate of metastases that it would be prohibitive to use that many animals 8 to see a difference. We switched to a model where we inject the bone fragments with tumor and implant them. We can follow growth with IVIS as the cells have luciferase. We also attempted to grow human dura with no good results. We can only procure adult human dura and it looks like it does not grow as well as immature dura. This will not allow us sufficient samples to perform experiments with human dura, but since the cytokines are very similar we have strong faith that the results would be similar.

10. Products

11. Participants & Other Collaborating Organizations: University of Michigan What individuals have worked on the project? Name: Sabrina Rocco Project Role: Laboratory Technician Researcher Identifier (e.g. ORCID ID): NA Nearest person month worked: 6 Contribution to Project: Ms. Rocco has performed the bench experiments Funding Support: NA Name: Nicholas Szerlip Project Role: PI Researcher Identifier (e.g. ORCID ID):

0000-0003-1116-3422 Nearest person month worked: 8 Contribution to Project: Dr. Szerlip has organized and set up experiments and oversees work on project Funding Support: NA Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? No