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How MMPs Impact Bone Responses to Metastatic Prostate Cancer

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How MMPs impact bone responses to metastatic prostate cancer

Using an animal model of prostate tumor progression in the bone we have previously shown that MMPs, namely MMP-2,-3,-9 and -13, are overexpressed at the tumor bone interface and these MMPs are for the most part expressed by the host cells of the bone. To test the contribution of MMPs in prostate tumor progression in the bone, we have generated mice that are immunocompromized and deficient for MMP-2,-3 and -9 during the current period. We have found that MMP-9 does not contribute to prostate tumor progression in the bone since no difference in osteolytic or osteoblastic responses between wild type and MMP-9 deficient animals were detected by Faxitron, CT, SPECT and histomorphometry. These results, while negative, are important for the generation of selective MMP inhibitors that lack the deleterious side effects associated with broad spectrum inhibitors. In addition, we have also identified PTHrP as an MMP substrate and postulate that MMP processing of PTHrP may be a mechanism through which MMPs can contribute to tumor induced osteolysis.

Osteolysis, osteoblastic changes, prostate progression in bone, matrix metalloproteinases, MMPs, receptor activator of nuclear kappa B ligand, RANKL, bone metastasis.

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This year, in the United States alone, of the 27,350 men who die from prostate cancer, 80% will have evidence of bone metastasis (1, 2). Prostate bone metastases cause several complications for patients such as hypercalcemia, spontaneous bone fracture and debilitating pain that dramatically affects their quality of life. To progress in the bone, the invading prostate tumor cells induce radical changes in bone matrix homeostasis by stimulating osteoblastic and osteolytic changes (3). These changes result in an actively remodeling bone tumor microenvironment, rich in mitogenic signals that promote tumor growth. In turn, the growth of the tumor exacerbates the osteoblastic and osteolytic changes in a manner that has been well described as the ‘vicious cycle’ (4). Using an animal model of
tumor progression in the bone, we have previously identified a group of enzymes known as matrix metalloproteinases (MMPs) as being highly overexpressed at the tumor bone interface in comparison to the tumor area alone. In a bid to understand the importance of these MMPs, namely MMP-2, -3, -9 and -13, in prostate tumor progression in the bone, we aim to generate MMP null animals and compare those animals to their wild type counterparts. While the MMPs are important in the turnover of the extracellular matrix, it has become apparent that the MMPs are also capable of regulating cell:cell communication by processing various cytokines and growth factors to active soluble forms (5). These soluble factors often influence biological processes including survival, proliferation, angiogenesis and osteoclast activation. Therefore, understanding which MMPs are important in contributing to prostate tumor progression in the bone and identifying the mechanisms that govern the vicious cycle can provide valuable targets for therapeutic development.

**Body..................................................................................................................**

**Accomplishments**

**Aim 1.** *Determine the stromal contribution of MMPs that are markedly overexpressed at the tumor:bone interface namely, MMP-2,-3,-9 and -13 to prostate cancer induced osteoblastic and osteolytic changes in the bone.*

a) Generate immunocompromized RAG-2\(^{-/-}\) mice that are deficient in MMP-2, MMP-3 and MMP-13 by crossing RAG-2\(^{-/-}\) mice with MMP\(^{-/-}\) mice that are both available on the C57Bl/6 background (Months 1-12).

b) Using our pre-clinical animal models, we will test the contribution of stromal MMP-9 to tumor induced osteoblastic and osteolytic change in readily available immunocompromized RAG-2\(^{-/-}\) MMP-9 deficient mice (Months 1-12).

c) Test the contribution of stromal MMP-2, MMP-3 and MMP-13 to tumor induced osteoblastic and osteolytic change using our pre-clinical model (Months 11-30).
d) Identify the expression of stromal MMPs in human clinical samples of prostate bone metastases (Months 20-36).

The proposed animal model used in the study involved the transplantation of moderately differentiated rat prostate adenocarcinoma to the calvaria of immunocompromized wild type and MMP deficient mice. To achieve this, we crossed C57Bl/6 RAG-2 (recombinase activating gene-2) deficient mice with either C57Bl/6 MMP-2, -3 or -13 deficient animals in order to generate F2/F3 animals that are immunocompromized and deficient for the desired MMP. As of January 2010, we generated RAG-2$^{-/-}$;MMP-2$^{-/-}$, RAG-2$^{-/-}$;MMP-3$^{-/-}$ and RAG-2$^{-/-}$; MMP-13$^{-/-}$ animals and have made preliminary observations with these animals in terms of prostate tumor induced osteolytic and osteoblastic changes.

*Host MMP-9 impacts angiogenesis in the prostate tumor-bone microenvironment but does not impact tumor induced osteolytic and/or osteoblastic changes.*

In the initial 12 months of the project, we focused on assessing the impact of host derived MMP-9 in tumor induced osteolytic and osteoblastic changes. We observed that the cell responsible for bone destruction, the osteoclast, was the major cellular source of MMP-9 in the prostate tumor-bone microenvironment in our animal model. The clinical relevance of this observation was also tested. In collaboration with Dr. Bob Vessella, University of Washington (6, 7), we assessed the expression of...
Fig. 2. Osteoclast derived MMP-9 regulates VEGF-A<sub>164</sub> bioavailability and angiogenesis. A, Representative photomicrographs of mature multinucleated osteoclasts generated from CD11b<sup>+</sup> myeloid precursor cells isolated from WT or MMP-9<sup>−/−</sup> mice after 10 days of culture in osteoclast differentiation media. Scale bars represent 50 µm. B, Quantitation of the number of osteoclasts generated by treatment with control or osteoclast (OCL) differentiation media. Only multinucleated (>3/cell) TRAcP positive cells were counted. C, Quantitation of VEGF-A<sub>164</sub> levels in conditioned media derived from WT or MMP-9<sup>−/−</sup> osteoclast (OCL) cultures in the presence or absence of 100 ng/ml recombinant MMP-9. D, Representative photomicrographs of mouse thoracic aortas stimulated with WT or MMP-9<sup>−/−</sup> osteoclast culture conditioned media (CM) at day 7. The average distance of sprout migration was calculated from photomicrographs on a daily basis. Scale bar represents 1mm. n.s. denotes non-significant (p>0.05) p values. * denotes p<0.05, ** denotes that p<0.01, *** denotes that p<0.001.

MMP-9 in 10 human samples of prostate to bone metastasis and observed that again, osteoclasts were a major cellular source of MMP-9 in the human prostate tumor-bone microenvironment. To test whether host MMP-9 contributed to prostate tumor induced osteolysis or osteogenesis, we utilized immunocompromized animals that were either wild type or null for host MMP-9. In repeated studies, with at least 10 animals per group, we determined that MMP-9 does not have any effect on tumor progression in the bone using both whole animal imaging modalities such as microCT and microSPECT and traditional histomorphometry approaches. While there was a trend towards a decrease in osteolysis in the MMP-9 deficient animals, this decrease did not prove to be statistically significant (Figure 1). While these data suggest that MMP-9 does not contribute to tumor progression in the bone, it should be stated that the role of MMP-9 in 1) the metastasis of prostate cancer to the bone or 2) in the initial survival/establishment of the prostate tumor cells in the bone microenvironment can not be ruled out since these steps are not recapitulated in our animal model. Previous studies have shown that MMP-9 is important in mediating angiogenesis in the tumor microenvironment (8) and using the endothelial antigen CD-31 as a marker for angiogenesis, we found decreased angiogenesis in the MMP-9 null group. In follow-up experiments we identified that while MMP-9 did not impact the ability of myeloid cells to become
mature multi-nucleated osteoclasts, there was a significant decrease in the ability of the MMP-9 null osteoclasts to mediate the bioavailability of the potent angiogenic factor vascular endothelial growth factor A (VEGF-A_{164}) (Figure 2). This observation explains, in part, the reduced vascularity of the tumors in the MMP-9 null animals. These results therefore demonstrate that while host, specifically osteoclast derived MMP-9, does not contribute to tumor induced osteolysis and osteogenesis, it does contribute to angiogenesis in the tumor-bone microenvironment. Furthermore, our results resonate with emerging studies from other groups that establish the osteoclast as an angiogenic cell (9, 10). These data have recently been reported in the journal *Molecular Cancer Research* (11).

**Host MMP-13 has a protective role in the prostate tumor-bone microenvironment**

Murine MMP-13 is considered to be the ortholog of human MMP-1, and MMP-13 deficient animals have been reported as having a delay in endochondral ossification during skeletal development with thickened trabecular bone persisting in the animals (12, 13). In collaboration with Dr. Stephen Krane, we generated immunocompromized MMP-13 deficient mice. Histological analysis of the calvaria from these animals at 6 weeks of age and their age matched wild type counterparts were similar with respect to the amount of bone and number of osteoclasts (data not shown). Surprisingly, preliminary studies using our animal model suggested that host MMP-13 plays a protective role in preventing tumor induced osteolytic and osteoblastic changes (Figure 2). At three weeks post implantation,
immunocompromized wild type and MMP-13 deficient animals were sacrificed. Histological analysis revealed that in comparison to wild type controls, MMP-13 deficient sections had higher numbers of osteoblasts and osteoclasts which was consistent with increase bone formation and destruction respectively. These data are unexpected since MMP-13 has been described as the rate limiting collagenase for murine skeletal development. These data suggested that in the pathological context of the tumor bone microenvironment, host MMP-13 inhibits the vicious cycle and plays a protective role in the tumor-bone microenvironment. The concept of MMPs as playing a protective role during tumor progression has been reported for MMP-3 and MMP-8 during skin carcinogenesis and progression in mice but to our knowledge MMP-13 has not been described as being protective in a pathological context (14, 15). These studies have been repeated and we are currently exploring the mechanisms through which MMP-13 protects against prostate tumor progression in the bone. Our collected data illustrated that MMP-7, -9 and -13 are highly expressed in the prostate tumor-bone microenvironment but on an individual basis, they clearly play very different roles with respect to tumor induced osteoblastic and osteolytic changes. In the final year of the proposal, we have also observed that host MMP-2 significantly impacts the progression of tumors in the bone microenvironment while host MMP-3 appears to play a limited role upon gross observations. Our currently research is focused on identifying how MMP-2 and MMP-13 can contribute to/or protect against prostate tumor progression in the bone microenvironment using our animal model. We anticipate that the results of these studies will be published within the next 12 months.

**Conclusions from Aim 1.** Initially we set out to test whether individual host MMPs contributed to prostate tumor progression in bone using a unique animal model. Our results and preliminary findings show that in some cases, individual MMPs can contribute (MMP-7 and MMP-2) while others can be protective (MMP-13) or play more subtle roles (MMP-9). These findings support the conclusions drawn from human clinical trials with MMP inhibitors that stated in order to avoid the deleterious side effects of broad spectrum MMP inhibitors, the roles for individual MMPs needed to be elucidated so
that more selective therapeutics could be generated. The data collected during this grant highlights the individual roles that MMPs can play in the prostate tumor-bone microenvironment and provides a shortlist of MMPs (MMP-2, MMP-7 and MMP-9) that could be therapeutically targeted for the treatment of prostate to bone metastases.

**Aim 2. Identify and test MMP processed substrates that mediate prostate tumor induced osteolytic and osteoblastic change.**

   a) Identify and test candidate MMP substrates that mediate prostate tumor induced osteolytic and osteoblastic change.

   b) Determine the contribution of MMP solubilized RANKL vs. MMP resistant RANKL in mediating osteoclastogenesis

In aim 2, we took a candidate approach in a bid to identify the potential factors that MMPs process in order to mediate tumor induced osteolysis. Bone is a rich reservoir of growth factors such as transforming growth factorβ (TGFβ) and insulin like growth factor-1 (IGF-1) and both of these factors have been implicated in driving the ‘vicious cycle’ (4). Given that various MMPs have been reported as processing the molecules that keep these growth factors in a latent state such as latency TGFβ binding proteins (LTBPs) and IGF binding proteins (IGF-BPs) we envisaged that these would be excellent candidate molecules through which MMPs could contribute to osteolytic and osteoblastic changes in the bone microenvironment.

**MMP-2 is a key regulator of TGFβ bioavailability**

In our initial studies, we examined the presence of active TGFβ using ELISA techniques. While we observed that there is more active TGFβ at the tumor bone interface in comparison to the tumor area alone, we found no difference in the levels of active TGFβ between wild type, MMP-7 and MMP-9 null animals (Figure 4). Given the labile nature of TGFβ we have taken multiple approaches into
identifying the activity status of the cytokine in wild type and MMP null animals. Using procedures described by Barcellos-Hoff et al. (16), we generated frozen sections of non-decalcified bone using the Cryo-Jane Tape transfer system. The approach allowed us to visualize latent and active TGFβ and the effectors of TGFβ such as phospho SMAD2 in the tumor-bone microenvironment using microscopy (Figure 4).

In parallel studies, we localized the major cellular sources of the MMPs under investigation in human and murine tumor-bone microenvironments. Surprisingly, we identified that osteoblasts and osteocytes are major sources of MMP-2. In Specific aim 1, our initial studies revealed that host MMP-2 contributed to tumor progression in the bone microenvironment. Thus far we have not generated enough animals to test whether differences in the levels of active TGFβ exist between the wild type and MMP-2 null immunocompromized animals. Therefore, in order to test if osteoblast derived MMP-2 is regulating the bioavailability of TGFβ we isolated primary osteoblast cultures from immunocompetent MMP null animals and tested the ability of the MMP null osteoblasts to produce active TGFβ in vitro. Our initial experiments have provided exciting results. We have identified that osteoblast derived MMP-2 plays a major role in facilitating TGFβ activation and for first time have shown that LTBP-3 which is predominantly expressed in the skeleton is a major player in this process. In our preliminary observations, we have found that osteoblast derived MMP-2 contributes to tumor progression in the bone microenvironment and that the regulation of TGFβ bioavailability is the major
molecular mechanism underlying this phenomenon (Figure 4). We are currently repeating these experiments using our animal model system but are also using other models in order to test the role of osteoblast derived MMP-2 in tumor progression in the bone microenvironment. We anticipate that the findings of these studies will be published by the end of 2010.

**PTHrP is a novel MMP substrate**

In the metastatic bone:tumor microenvironment, parathyroid related hormone (PTHrP) has been identified as a powerful mediator of osteolysis (17). Pro-PTHrP has three isoforms that are 139, 141 or 173 amino acids in length. These isoforms are subsequently enzymatically processed to yield the mature form of PTHrP1-36 (amino acids 1-36). Thus far the enzymes implicated in generating mature PTHrP have been; endothelin converting enzyme-1 (ECE-1); ECE-2 and neprilysin which are not MMPs but are members of the metazincin family of proteinases. Interestingly, prostate specific antigen (PSA) which is a serine protease has also been shown to process PTHrP but in a different region that generates a 23 amino acid form of PTHrP1-23 (18). This is thought to abolish the activity of the hormone but some studies suggest that smaller molecular weight versions of PTHrP can have differential effects compared to PTHrP1-36 (19).

![Fig. 5. PTHrP is an MMP substrate. A: RT-PCR analysis of PTHrP expression by the prostate cancer tissue (PLSP). +ve refers to positive control. B: Mature PTHrP (100ng; arrow head) was incubated for 1 hour at 37°C either alone or with 100ng of active MMP-3. Arrow indicates cleavage product. Markers are in kDa. C: Immunoblot analysis also demonstrates PTHrP processing using an antibody directed to the C-terminus (Santa Cruz; sc-20728). D: N-terminal amino acid sequencing revealed that MMP-3 generated PTHrP1-36. F, MALDI-TOF-MS showed that MMP-3 was capable of further processing of PTHrP1-36. Similar analyses revealed no degradation of full length PTHrP in the absence of MMPs. E-F: MC3T3-E1 (10^5 cells per 6 well in triplicate) were incubated with 50nM PTHrP1-36 or equimolar concentrations of PTHrP fragments corresponding to MMP-3 products. No difference in mineralization was determined by day 21 using alizarin red and spectrophotometry (E). However, treatment with PTHrP1-16 significantly stimulated MC3T3 cell growth as assessed by MTT assay (F).](image-url)
Since PTHrP can be processed by members of the metazincin family and given the presence of MMPs in the tumor bone microenvironment, we asked whether MMPs could process PTHrP. Using recombinant PTHrP\textsubscript{1-141}, we observed that MMP-3 and MMP-7 generate mature PTHrP\textsubscript{1-36} (Figure 5). Further examination by mass spectroscopy revealed that MMP-3 and MMP-7 further processed PTHrP\textsubscript{1-36} into smaller fragments, namely PTHrP\textsubscript{1-16}, PTHrP\textsubscript{17-26} and PTHrP\textsubscript{27-34}. A number of the MMP generated PTHrP products have reported cellular functions. For example, PTHrP\textsubscript{1-16} has sequence similarities to endothelin-1 (ET-1). ET-1 has been identified as a major factor involved in promoting osteoblastic responses via the ET\textscript{A} receptor (20). PTHrP\textsubscript{1-16} has been shown to bind to ET\textscript{A} in cardiomyocytes but apparently has no impact on ET\textscript{A} signaling when overexpressed in CHO cells (21, 22). However, the precise role of PTHrP\textsubscript{1-16} in osteoblast function is unclear. Other MMP-3 generated fragments such as PTHrP\textsubscript{27-36} can mediate protein kinase C signaling via the PTHR-1 receptor (23) but again, the precise role of this fragment in addition to PTHrP\textsubscript{17-26} in osteoblast function remain unclear. Our initial experiments identified that the MMP generated fragments can impact the behavior of the osteoblasts with respect to growth but it does not appear that the fragments impact osteoblast differentiation and invasion. We are currently further assessing the in vitro and in vivo relevance of the MMP generated PTHrP products and they will be the focus of future studies.

**MMPs generate an active soluble form of RANKL.**

We have previously demonstrated that membrane bound receptor activator of nuclear \(\kappa\)B ligand (RANKL) which is essential for osteoclast maturation and activation is sensitive to shedding from the cell surface by MMP-3 and MMP-7 (24) and have found that the mechanism is not only relevant in the prostate tumor-bone microenvironment but also in the breast (25). Experiments in this

![Fig. 6. A: MMP-7 processes RANKL in the juxtamembrane region at amino acid position 142 (see figure 10 for additional information). To generate a non-cleavable mutant RANKL, the methionines at positions 142 and 143 in addition to the proline at position 140 were mutated by single nucleotide substitution using a site directed mutagenesis kit (Stratagene). Methionine to Valine (ATG to GTG) and Proline to Leucine (CCA to CTA) B: Wild type and \(\Delta\)-RANKL full length cDNas were in vitro translated using the Promega TnT \textit{in vitro} translation system with S\textsuperscript{135} labeled methionine. 1: \(\Delta\)-RANKL alone. 2-3: \(\Delta\)-RANKL(2) or wild type RANKL(3) incubated with 100ng of MMP-7 overnight at 37°C. Arrow indicates full length unglycosylated \(\Delta\)-RANKL at approx 40kDa. Arrow heads denote cleavage products.](image)
study have resulted in the generation of non-cleavable of RANKL and future studies are focused on testing the ability of the non-cleaved RANKL to stimulate osteoclast activation via direct cell:cell contact and generating knock in animals (Figure 6).

Conclusions from Aim 2

Using a candidate approach we have found that MMPs that are highly expressed in the tumor-bone microenvironment (MMP-2, -3, -7) can process the factors that drive the vicious cycle. MMPs have primarily been considered as playing important roles in matrix degradation and while this may be true, clearly they are also playing key roles in regulating the availability and bioactivity of key factors such as PTHrP, RANKL and TGFβ. These discoveries have several innovations and highlight potential areas for therapeutic targeting.

Key Research Accomplishments………………………………………….…………………

- Generated RAG-2;MMP-2, RAG-2;MMP3, RAG-2;MMP-13 null animals
- Identified osteoclasts as a major source of MMP-9 in the human and murine prostate tumor-bone microenvironments
- Demonstrated that host derived MMP-9 does not contribute to tumor progression in the bone but does impact angiogenesis
- Determined that host MMP-13 plays a protective role in preventing tumor induced osteolytic and osteoblastic changes
- Identified that host MMP-2 contributes to tumor progression in the bone microenvironment
- Identified higher levels of TGFβ at the tumor bone interface in comparison to the tumor area alone in wild type animals
- Identified that osteoblast derived MMP-2 is a major regulator of TGFβ bioavailability
- Identified that MMP-3 and MMP-7 are capable of generating mature PTHrP
- Identified that MMP generated PTHrP fragments impact osteoblast behavior
• Generated a non-cleavable version of RANKL

Reportable Outcomes..............................................................................................................

Manuscripts

Book Chapters
Presentations

Tumor Microenvironment Steering Committee Meeting, Nashville, TN, 2009.


Growth Factor and Signaling Symposium, Ames, Iowa, September, 2008

Joint AACR and Metastasis Research Society Meeting, Vancouver, BC, Canada, August, 2008

Tumor host interaction and angiogenesis meeting, Monte Verita, Ascona, Switzerland, October 2007.

Tumor microenvironment (TMEN) meeting, Vanderbilt University, Nashville, TN, September, 2007.

Grants

NCI-(1RO1CA143094-01A1). 01-JUL-10-30-JUN-15.

Role: Principal Investigator.

Title: Host MMP-mediated regulation of the vicious cycle of prostate to bone metastases

Overall Conclusions

Our results have shown that individual MMPs can have differential effects in the tumor-bone microenvironment. Previously, MMP inhibitors failed in the clinical setting, primarily due to a lack of understanding as to how MMPs contribute to tumor progression. In animals models of osteolysis, broad spectrum MMP inhibitors have been successful in preventing tumor induced osteolysis and growth (26-28). Therefore, in order to apply MMP inhibitors in the clinical setting, our results suggest that the selective targeting of MMP-2, MMP-7 and MMP-9 while sparing the activity of other metalloproteinase family members such as MMP-13 would be of benefit for the treatment of prostate to bone metastases. Finally, as a new investigator award mechanism, this grant from the DOD has been essential in allowing me to become an independent and established investigator. Based on my results acquired from this application, I have been able to generate an National Cancer Institute RO1 proposal that recently scored in the 12th percentile and is highly likely to be funded beginning July 1st.
2010. Therefore, I am extremely grateful for the DOD in allowing me this opportunity to kick start my career in identifying cures for prostate to bone metastases.

References............................................................................................................................................


