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14. ABSTRACT Hyaluronan is a high molecular weight polyanionic polysaccharide that is increased in more advanced prostate cancers. Tumor cell interaction with this polysaccharide by specific receptors CD44 and RHAMM promote tumor growth, survival and invasion. Work during the last funding period have further defined the mechanism of action of each of these receptors. Studies show that extracellular RHAMM acts a co-receptor for CD44, and the combined action of this receptor complex leads to sustained activation of the ERK 1,2 signal transduction pathway leading to enhance motility and produce patterns of gene transcription that are associated with invasion. Synthetic peptides have also been identified that can bind hyaluronan and inhibit the binding of this polysaccharide to its cognate receptors. These peptides inhibit tumor growth both <i>in vitro</i> and <i>in vivo</i> and the residues important for the activity of the peptides are being defined using nuclear magnetic resonance (NMR). Small molecule libraries that contain compounds which may mimic these peptides are also being interrogated for the ability to inhibit hyaluronan binding to RHAMM and CD44 and to inhibit tumor growth. The goal is to develop new therapeutic strategies for patients with invasive prostate cancer.					
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Introduction:

The last year of funding has been largely dedicated to generating specific antibodies and synthetic peptides that were be used during year 4 (no cost extension). The activities during year 4 are highlighted in red text.

The focus of this grant is to evaluate the importance of hyaluronan and tumor cell receptors for hyaluronan in promoting tumor progression. Hyaluronan is a high molecular weight (10^5 - 10^7 daltons) anionic carbohydrate that is associated with the progression of a number of cancers, including prostate cancer (Simpson and Lokeshwar, 2008; Toole, 2004). Tumor associated hyaluronan first appears in the tumor reactive stroma, whereas later stages of progression are also associated with an increase in synthesis by the epithelial compartment of the tumor (Simpson and Lokeshwar, 2008). Furthermore, tumor associated hyaluronan is also fragmented, in part by an upregulation of hyaluronidases, which create smaller oligomers that have different biological properties than the high molecular weight polymer. One important difference in the biological properties of lower molecular weight oligomers ($\sim 3 \times 10^3$ daltons) is that such oligomers will stimulate angiogenesis, whereas higher molecular polymers impede angiogenesis. Furthermore, hyaluronan/tumor cell interactions enhance tumor cell survival, drug resistance and increase both growth and invasion by mechanisms that are not yet completely understood (Toole, 2004; Toole et al., 2005). Importantly, strategies to disrupt tumor cell/hyaluronan interactions do offer the potential to impede tumor progression and enhance sensitivity to current therapies. This is the underlying rationale for pursuing the studies funded by this grant award.

The two hyaluronan receptors expressed by invasive/metastatic prostate tumor cells are CD44 and RHAMM (CD168), and the funded studies focused on understanding the relative importance of each receptor in the biology of prostate tumor progression. There have been substantial changes to the understanding of RHAMM function since this initial award was made. We summarized some of these results in a recent opinion paper published by the PI and co-PI in Journal of Cell Science.((Maxwell et al., 2008)). This JCS paper documents the history of studies of RHAMM function, which include its original discovery as an extracellular motogen receptor for hyaluronan (Turley et al., 2002). The paper also highlights other studies demonstrating that RHAMM has intracellular functions, which include binding to interphase microtubules, localizing within the nucleus, and participating in mitotic spindle formation (Assmann et al., 1999; Maxwell et al., 2003; Turley et al., 2002). Dysregulation of intracellular RHAMM levels in cells has also been proposed to contribute to genomic instability in cells (Joukov et al., 2006; Pujana et al., 2007). We also recently finalized a chapter for Seminars in Cancer Biology that summarizes the entirety of our findings that were in large part funded by the DOD award. Two additional peer reviewed papers, which have been funded by the DOD award, have also also been published during the funding period (Tolg, et al. 2006; Hamilton, et al. 2007). In these studies, models in addition to prostate cancer have been characterized for RHAMM function since each model has its advantage compared to the prostate cancer cell lines being used in the funded award. These studies have a direct bearing on changes in the original design of studies to use prostate cancer cell lines in these studies. One of

these papers utilizes a RHAMM -/- animal model to specifically address the function of extracellular RHAMM, since the focus of the current prostate cancer proposal is to address the hyaluronan related properties of this motogen. The second paper utilized breast cancer model cell lines that differ in the expression of cell surface RHAMM and cell surface hyaluronan. The conclusions from these studies have been essential for creating a testable working model for RHAMM function in prostate.

The funded proposal has three specific aims which are:

Specific Aim 1: To evaluate the ability of hyaluronan to enhance tumor growth and invasion in the presence or absence of CD44 and/or RHAMM.

Specific Aim 2: To identify structural features of CD44 and RHAMM that mediate the effects of hyaluronan on signaling pathways regulating tumor growth, survival and invasion.

Specific Aim 3: To test the ability of specific hyaluronan-binding synthetic peptides to inhibit hyaluronan binding to CD44 and RHAMM or tumor growth/survival in vitro and in vivo.

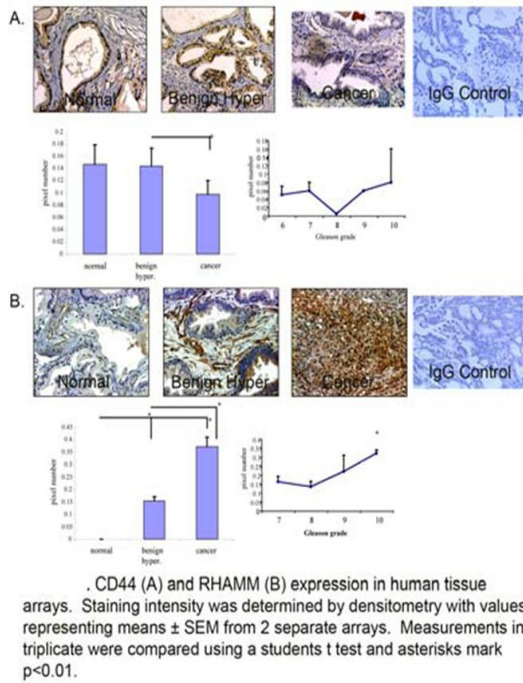
The next section addresses the state of progress on these studies as organized by the original Statement of Work.

Body

Statement of Work:

Aim 1: to evaluate the ability of hyaluronan to enhance tumor growth and invasion in the presence or absence of CD44 and/or RHAMM

Task 1: Determine the effect of inhibiting CD44 or RHAMM function/expression on stimulating the growth, invasion and motility of PC3M-LN4 cells.

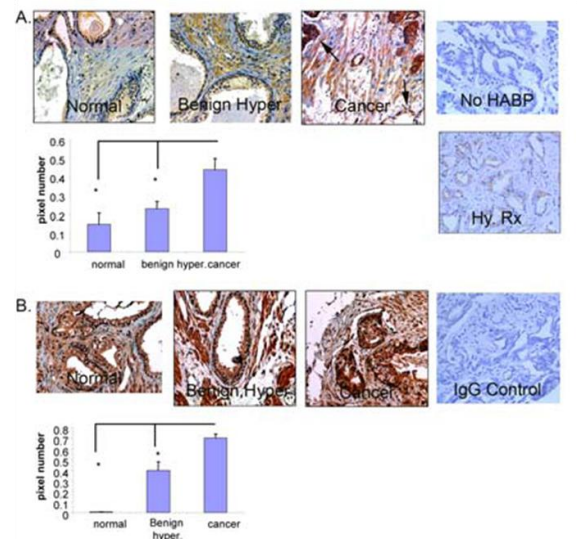


Although a study of human tissues was not originally part of the work statement, comments were made during the review that relevance to human disease had not been clearly established, and so we have first embarked on a more systematic study to evaluate the relationship between CD44, RHAMM, hyaluronan (HA) and hyaluronidase 1 expression (Hyase 1).

Human tissue microarrays were used to first evaluate the levels of CD44 (Figure 2A), RHAMM (gene name, HMMR; Figure 2B), HA (Figure 3A) or Hyal-1 (Figure 3B) in human tumors. The tissue microarrays each had 40 samples equally divided between benign hyperplasia (BPH) and different Gleason Grades (6-10), along with two normal controls. Anti-RHAMM antibodies were generated against a synthetic peptide from residues 169 to 182 (ELMKLRNKRETMR) and the IgG purified from immune or prebleed sera as described (Appendix 2 and 3) and specificity was confirmed by Western blot of PC3M-LN4 cell extracts vs. RHAMM +/- fibroblast extracts or recombinant RHAMM protein (not shown). The tissue sections on the microarray were scored for Gleason Grade and initially the staining intensity of both tumor stroma and epithelium were ranked by Dr. Steve Schmechel (surgical

pathologist, U of MN), then intensity in the epithelial compartment was quantified using image analysis and densitometry.

The level of tumor associated CD44 staining overall was reduced in cancer samples compared to normal tissue or BPH and it remained lower but did not vary as a function of Gleason score (Figure 2A). In contrast, RHAMM was not detected in normal tissue but was elevated in BPH, and most strongly elevated in prostate carcinomas (Figure 2B). The intensity of RHAMM staining increased as a function of Gleason Score (Figure 2B). Elevated levels of both HA (Figure 3A) and Hyal-1 (Figure 3B) were also observed in both BPH and prostate cancer, with a statistically higher staining intensity detected in the carcinoma compared to normal or BPH epithelia. The staining intensity of both HA and Hyase-1 also increased as a function of Gleason Scores (not shown). Dr. George Vasmatazis (Bioinformatics, Mayo Clinic) has also provided data obtained using microarray expression analysis of laser capture microdissected tumors comparing normal and tumor cells within individual tumors (personal communication). His initial findings from the microarray data indicate that RHAMM expression in the carcinoma increases is associated with aggressiveness (p -value 0.04) and is independent of Gleason



Score. Furthermore, RHAMM expression is very high in 6/7 metastases (nodal metastasis cases), implicating the expression of this gene in malignant progression. Collectively, these data, and the studies summarized below (included in the Appendix), strongly support the rationale for evaluating the cooperation of RHAMM and CD44 in a hyaluronan/hyaluronidase rich environment in primary or metastatic human prostate tumors.

Studies have been initiated to evaluate the relative importance of RHAMM and CD44 in promoting prostate tumor cell migration. Some of these studies have utilized a RHAMM $-/-$ mouse, generated by Dr. Turley ((Tolg et al., 2006), Appendix 2). The reason for utilizing this mouse model, developed by Dr. Turley, is related in part to the realization that RHAMM, which functions as an extracellular hyaluronan receptor, also has intracellular functions which include binding to microtubules, participating in mitotic spindle assembly and it can also localize to the nucleus (Assmann et al., 1999; Maxwell et al., 2003; Maxwell et al., 2008; Turley et al., 2002). Indeed, recent studies by Livingston's group have implicated dysregulation of RHAMM levels (due to defects in BRCA1 E3 ubiquitin ligase activity) in abnormal spindle formation (Joukov et al., 2006; Pujana et al., 2007).

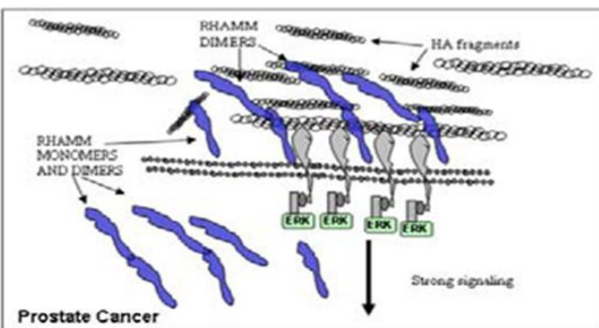
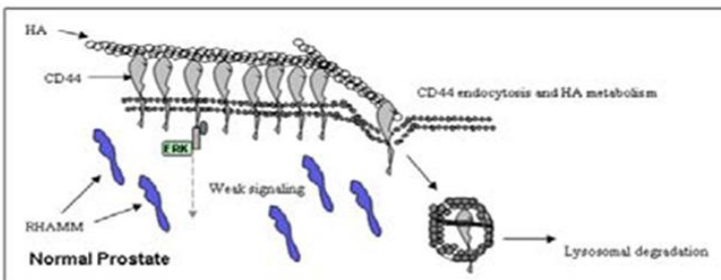
The conclusions of the published knockout animal studies are summarized as follows:

1. RHAMM $-/-$ animals exhibit defects in excisional wound healing.
2. The inflammatory response is abnormal in wound of RHAMM $-/-$ animals
 - a. The granulation tissue of RHAMM $-/-$ wounds is abnormal compared to wild type controls
 - b. Neutrophils persist longer than in wild type animals
 - c. There is a defect in monocyte/macrophage infiltration into the granulation tissue
3. Fibrogenesis/repair in RHAMM $-/-$ animals is also defective
 - a. Fibroblasts from RHAMM $-/-$ have defective migratory properties both in vivo and in vitro
 - b. The sustained activation of ERK 1,2, which has been associated with extracellular RHAMM function, does not occur in RHAMM $-/-$ fibroblasts in vivo or in vitro
 - c. The defective phenotype can be rescued with beads to which recombinant RHAMM has been attached. This was used to avoid complications of internalization of RHAMM in the RHAMM $-/-$ cells. The rescued motility phenotype was sensitive to inhibition by MEK1 inhibitors
 - d. Recombinant RHAMM coupled beads also rescued sustained activation of the ERK 1,2 pathway leading to nuclear localization of pERK 1,2
4. Extracellular RHAMM mediates motility and sustained activation of the ERK 1,2 pathway by enhancing cell surface localization of CD44
 - a. CD44 colocalizes with RHAMM coated beads on the surface of RHAMM $-/-$ fibroblasts
 - b. Antibodies against CD44 inhibit wild type fibroblasts inhibits hyaluronan stimulated motility. These antibodies also inhibit the enhance migration of RHAMM $-/-$ fibroblasts stimulated with RHAMM coated beads
 - c. RHAMM $-/-$ CD $-/-$ cells do not enhance migration in response to HA and are not rescued by RHAMM coated beads

Therefore, we conclude from this study that extracellular RHAMM can stimulate motility in the absence of intracellular RHAMM, and that extracellular RHAMM works in conjunction with CD44 to stimulate motility and enhance the intensity and duration of activation of the ERK 1, 2 pathway. We hypothesize that this RHAMM-enhanced activation of ERK 1, 2 via CD44 is at least partly due to enhanced cell surface retention of CD44.

A second publication credited to the DOD award published in J. Biol. Chem. (Hamilton et al., 2007) utilized well characterized breast tumor cell lines for motility and RHAMM expression. Two paired cell lines with differing invasive properties were compared for RHAMM, CD44 and HA expression. The studies have led to our constructing a model for RHAMM, CD44, hyaluronan and ERK 1, 2 activation in prostate cancer cells. The findings in the J. Biol. Chem. paper are as follows:

1. Highly motile/invasive breast cancer cell lines MDA-MB-231 or Ras transfected MCF 10A cells express cell surface RHAMM, CD44, hyaluronan and exhibit sustained activation of the ERK 1,2 pathway compared to poorly invasive/migratory MCF7 or parental MCF 10A cells.
2. RHAMM, CD44 and active ERK 1, 2 co-precipitate from the highly migratory cell lines indicative of a macromolecular signaling complex.
3. Combinations of antibodies against CD44, RHAMM or a MEK1 inhibitor were less than additive at blocking motility, suggesting all three are involved in a common motogenic pathway
4. Although not specifically tested in this system, the presence of extracellular hyaluronan in the highly migratory cells and the common hyaluronan binding properties of both CD44 and RHAMM have led us to propose that CD44 can pair, via hyaluronan, to cell surface RHAMM to form a signaling complex. A schematic of that model is shown below:



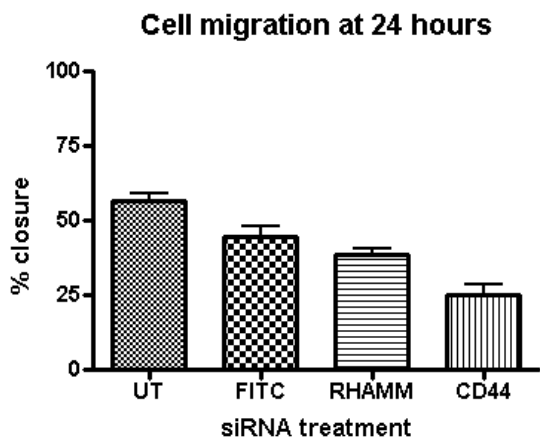
Proposed model for roles of CD44/RHAMM/HA interactions for integrating activation of signaling cascades that promote and sustain prostate tumorigenesis. In normal prostate tissue (A) CD44 protein is highly expressed and acts to maintain low levels of HA in the prostate cell microenvironment amongst other functions. RHAMM is intracellular and interacts with microtubules (not shown) (B) Upon malignant transformation, RHAMM is expressed and exported to the cell surface as monomers and dimers (which from the association from leucine zippers shown in Figure 1). RHAMM surface display of CD44 thereby blocking HA uptake and metabolism. HA fragments accumulate and bind to RHAMM (but not CD44.) The resulting complex enhances surface retention of CD44 hyperactivating CD44-related signaling pathways.

This model has certain predictions that are currently being tested.

1. Co-expression of RHAMM and CD44, as is observed in the highly metastatic PC3M-LN4 cell line, should form a macromolecular complex with ERK 1, 2 that can be detected by co-precipitation. Studies are currently underway to test this model in the prostate cancer model.
2. RHAMM expression should enhance the level of surface CD44 on these cells. The first studies involve using siRNAs against CD44 to limit expression of each and then to evaluate the amount of surface RHAMM remaining following CD44 knockdown. Secondly, RHAMM will be knocked down using RNAi and the level of CD44 remaining will be measured using Western blot and flow cytometry (to determine the surface level of CD44).
3. Inhibiting CD44 expression should limit association of RHAMM with ERK 1, 2 (as assessed by co-precipitation) and the activation state of ERK 1, 2 should be reduced.
4. Finally, limiting HA expression by the PC3M-LN4 cells (which synthesize and capture large amounts of HA on the cell surface) would be predicted to disrupt this complex. This will be accomplished by using hyaluronidase pretreatment or by using RNAi for the hyaluronan synthase enzymes expressed by these cells. The prediction, again, is that limiting HA production will inhibit cell surface RHAMM retention (and possibly inhibit surface levels of CD44).

Milestones and Outcomes: Evaluation of inhibitory antibodies for CD44 or RHAMM in anchorage independent growth, HA mediated motility/invasion of PC3M-LN4 cells in vitro and subcutaneous tumor growth in xenograft injections

Methods: CD44 and RHAMM expression/function are inhibited both in vitro and in vivo using defined antibodies and specific siRNAs and by stable transfection with conditional expression vectors coding for specific shRNAs. Specific inhibitory antibodies for RHAMM or CD44 are available from both the Turley Laboratory and the Naor Laboratory (see enclosed letter). TET-on conditional anti-CD44 or anti-RHAMM and negative control shRNA expression vectors were purchased from Genscript (Piscataway, NJ). shRNA expression is under the control of the human H1 shRNA promoter containing a tetracycline operator that, in the presence of tetracycline repressor (TetR) inhibits transcription. The vector also contains a coral GFP cDNA under a constitutive promoter, which is used for selecting transfected cells. Initially, tumor cells are transfected with vector pCDNA6/TR (Invitrogen), which constitutively expresses the TetR. Clones that demonstrate high TetR expression are then transfected with the inducible shRNA expression vectors and selected for GFP expression by FACSTM. CD44 and RHAMM specific antibodies and siRNAs will be evaluated for their ability to inhibit HA mediated motility and invasion using standard assays available in both the Turley and McCarthy laboratories (time lapse videography, modified Boyden chamber assays). Antibodies will be used to further evaluate the relative contribution of the HA interaction with CD44 or RHAMM in promoting anchorage independent growth in vitro of the metastatic PC3M-LN4 cells. PC3M-LN4 cells stably transfected with conditional shRNA vectors will be utilized to determine the impact of inhibiting CD44 and RHAMM expression on tumorigenic potential by subcutaneous injection or orthotopic injection into the prostate.



Studies have been initiated to determine the effect of inhibiting CD44 expression and function on PC3M-LN4 cell motility (Figure below). The assay used for this experiment is a scratch wound assay in which confluent tumor cell cultures are first treated with CD44 siRNA, RHAMM siRNA, or negative control FITC, incubated for 24 hours, and then the medium is removed and the cultures are wounded with a micropipette tip. The percent closure (relative to the width of the wound at time 0) is calculated from photomicrographs of the assay, thus the smaller the bar, the more effective the inhibitor. The CD44 siRNAs used in this assay effectively and significantly inhibit migration by over 50%, and the knockdown of CD44 protein has been verified (~80%, not shown). The RHAMM siRNA was not significantly inhibitory compared to the FITC control, and analysis of RHAMM

protein indicated this siRNA was not effective at inhibiting the level of RHAMM protein. We are in the process of screening additional siRNAs (from Qiagen) for RHAMM to identify those that are effective. The CD44 siRNA treated cells are also in the process of being further characterized for migration and anchorage independent growth.

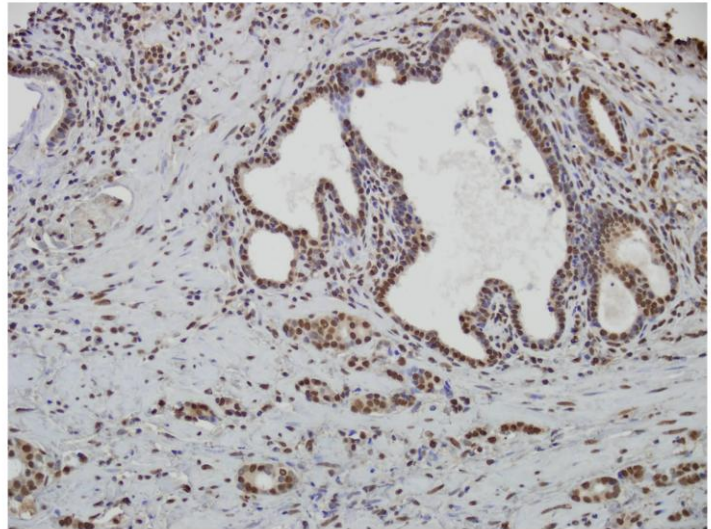
- a. Hyaluronan synthesis and Hyaluronan Synthase expression is being characterized for siRNA treated cells. We have spent the last year generating specific antibodies against specific HAS isoforms. These antibodies were raised against specific carboxyl terminal regions of all three HAS isoforms. We synthesized synthetic peptides from these regions and used them to generate polyclonal antibodies in rabbits. These antibodies were characterized by western blots and shown to be cross reactive immunofluorescence in cell and immunohistochemistry of human tissue sections. This will be completed in the next 3-4 months of year 4 and they will be used to evaluate the level of HAS isoforms following siRNA treatment.
- b. Expression and Surface Levels of both CD44 and RHAMM are also being analyzed. The prediction is that RHAMM surface levels will decrease with lower levels of surface CD44. **We had also contracted for the generation of specific monoclonal antibodies generated against the**

carboxyl terminal coiled coil domain of RHAMM protein. We have received 10 clones that were characterized for reactivity against human RHAMM. These antibodies were tested using

Rhamm null and Rhamm wild type fibroblast cell lines and shown to be specific for Rhamm (not shown).

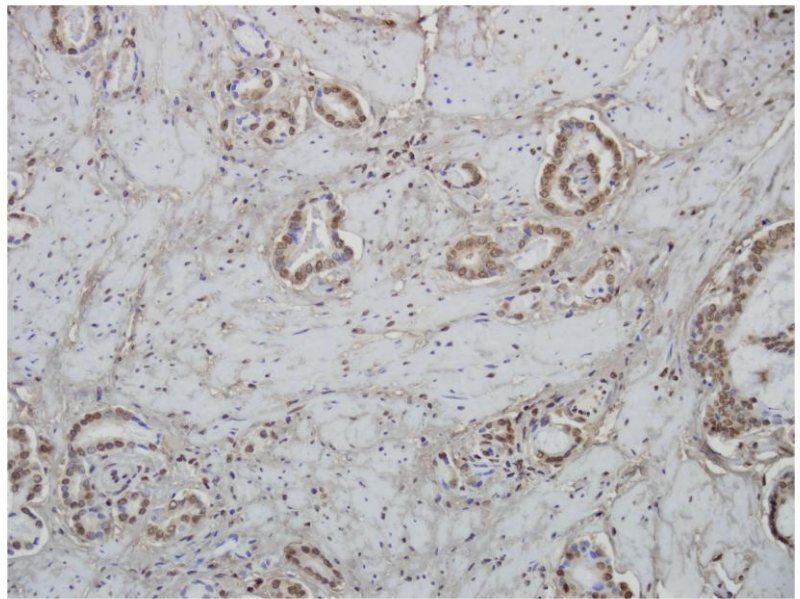
The antibodies were then characterized in

Immunohistochemistry of human prostate cancer tissue specimen. The images show that Rhamm staining is nuclear and cytoplasmic in epithelium (cancer and spotty in benign), nuclear in stroma, additionally in stroma there are “rivers” of Rhamm that correspond to loose connective tissue—the type sure to be HA rich . It is also detected in leukocytes within the stroma. These antibodies are being



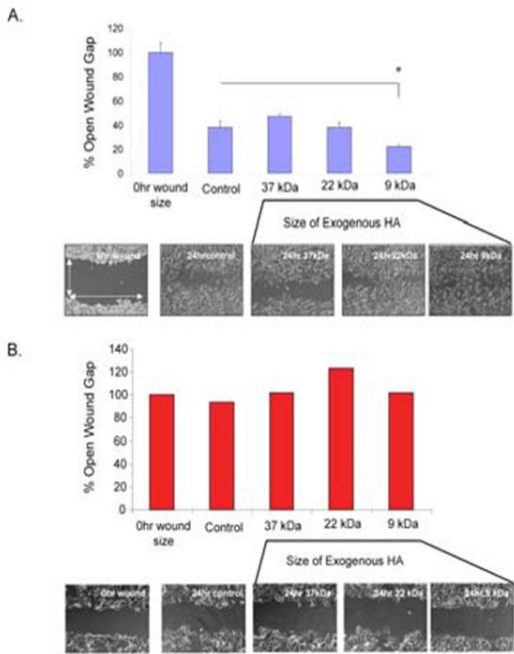
used in conjunction with anti-CD44 antibodies for double staining of a tissue microarray of cohorts with well characterized tissue microarrays to evaluate the potential prognostic significance of each recaptor alone or in combination. We will also use these antibodies to quantify RHAMM levels on the surface of tumor cells following

CD44 siRNA treatment of these tumor cells. The prediction is that co-staining of these two HA receptors will have prognostic value in terms of biochemical failure of the tumors. The surface level of RHAMM and CD44 will also be evaluated following specific inhibition of HAS isoform expression/HA synthesis by tumor cells. The prediction is that RHAMM levels (and possibly CD44) will decrease following inhibition of HA synthesis in PC3M-LN4 tumor cells



Task 2: Evaluate hyaluronan size distribution using FACE analysis of tumors grown as a result of subcutaneous injection of prostate cancer cells.

Time-Line: year 1



Effect of different HA fragment sizes on wound gap closure by human CaP cells. Migration of serum starved PC3M-LN4 cells (A) or 22RV1 (B) into gaps of scratch wounds. Monolayers were measured in the presence of 37 kDa, 22 kDa, or 9 kDa HA (LifeCore). Wound closure was measured as the size of cell-free wound gap remaining at 24 hours/size of original wound gap. Only 9 kDa HA significantly stimulated migration of PC3M-LN4 cells in this assay. Significant differences are marked by asterisks ($p < 0.01$). Values are the Mean and S.E.M (n=3 replicates) in the case of PC3M-LN4 and Mean (n=1) alone for 22RV1.

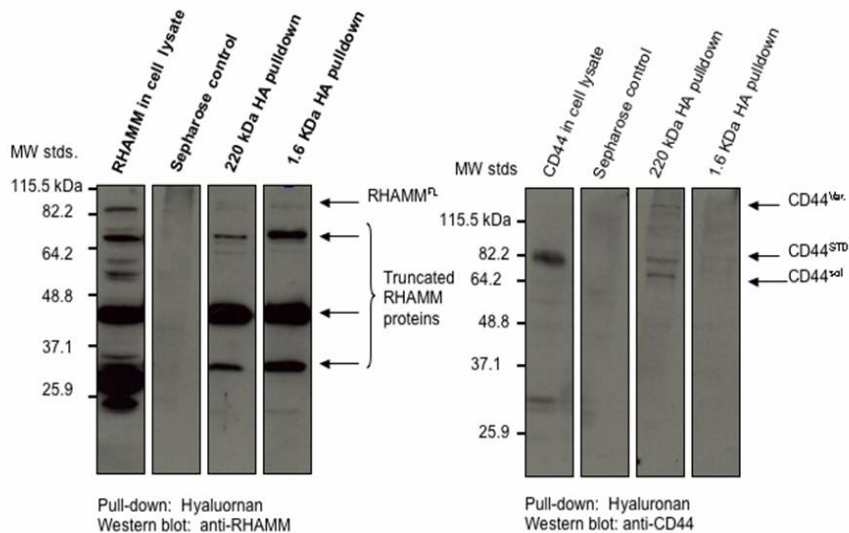
Milestones and Outcomes: Qualitative and quantitative evaluation of HA fragments produced by tumors developed as a result of subcutaneous injection of prostate cancer cells into immuno-compromised mice. The results of this task will determine which size of HA fragments will be used in ITC binding studies.

Methods: Fluorophore-assisted Carbohydrate Electrophoresis (FACE) can be used to determine the amount as well as size of hyaluronan produced in tissues or by cells in culture (Calabro A., et al. *Glycobiology* 2000; 10: 283-293, Calabro A., et al. *Blood*. 2002; 100: 2578-2585) and was recently established in the laboratory of E. Turley.

As a first step for this task, hyaluronan oligomers of defined size have been used in assays to define their biological properties. These defined oligomers have been obtained from Seikigaku (Japan) or Life Core (Chaska, MN). The motility promoting activity of these fragments has been evaluated using PC3M-LN4 cells (which express both CD44 and RHAMM) and for 22 RV1 cells (which express low to undetectable levels of both CD44 and RHAMM). The assay used is a scratch wound assay, where, as above, confluent tumor cells in culture are wounded with a pipette, and the closure of these wounds within 24-48 hours is evaluated. The cultures are then photographed and the distance remaining until wound closure is quantified. Data are presented as percent of the gap that remains to be closed by comparing the values with distance at time 0. Thus, in this case larger bars represent slower migration relative to smaller bars. The results of

the initial experiments are shown in the Figure above. The hyaluronan oligomers tested ranged from 9kDa to 37kD. Migration was stimulated by the addition of 9kD oligomers compared to that observed in the absence of oligomers, however the larger size HA oligomers failed to stimulate migration over that observed in the control cultures. As expected, the HA oligomers had no detectable effect on the migration of 22RV1 cells.

Our published studies using RHAMM^{-/-} cells and breast cancer cell lines with different levels of cell surface RHAMM indicated that extracellular RHAMM can act as a co-receptor for CD44. Based on these studies, we conclude that these co-receptors can form a signal transduction complex to sustain activation of the ERK 1, 2 pathway. One hypothesis is that different size hyaluronan oligomers will stimulate motility by selectively interacting with cell surface RHAMM. To test this hypothesis, beads coupled to



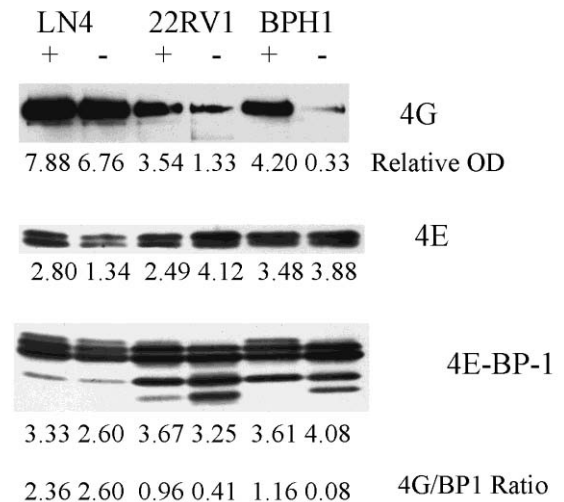
Hyaluronan-Sepharose pulls down RHAMM and CD44. PC3LN4 cell lysates were incubated with high molecular HA-sepharose (220kDa) and low molecular weight HA (960 kDa) columns. Bound RHAMM and CD44 were detected in western blots. Cell lysates were incubated with blocked sepharose columns as controls. RHAMM proteins (full length (FL), 85kDa) and truncated proteins (70, 45, 30 kDa) bound to both high and low MW HA, although more Rhamm protein bound to low MW HA. CD44std (85kDa) variants (120kDa) and soluble (60kDa) bound to high MW HA only but little if any bound to low MW HA. Neither RHAMM nor CD44 protein bound to Sepharose alone. These results are consistent with an ability of High MW HA to "bridge" an association between CD44 and RHAMM.

either small (1.6 kDa) or large (220 kDa) hyaluronan oligomers were used to pull down CD44 or RHAMM from the PC3MLN4 cell extracts (Figure above). The results demonstrate that RHAMM interacts with both sizes of hyaluronan oligomers (Left Panel), whereas CD44 only binds to the larger polymer (Right Panel). The results are consistent with a model in which smaller hyaluronan oligomers stimulate motility by selectively binding RHAMM. Since later stage prostate tumors express more RHAMM and increased levels of hyaluronidase, the studies implicate RHAMM in stimulating motility/invasion of later stage prostate tumors.

Our next step in these studies will be to use defined HA fragments to stimulate prostate tumor cells that express CD44 or surface RHAMM. The readouts for these studies include motility and activation of Erk 1,2 pathway and of Akt in the tumor cells. Both of these pathways have been linked to both CD44 and RHAMM stimulation and the prediction is that low molecular weight HA will function to stimulate cells that express cell surface RHAMM (which will be blocked with anti-RHAMM antibodies). In contrast, high molecular weight HA will signal via CD44 and possibly RHAMM (again assessed using specific blocking antibodies to each receptor).

These studies were put on hold to pursue an unexpected finding in terms of the potential importance of CD44 in the biology of prostate tumor progression. 5'-Cap dependent translation is known to be dysregulated in many tumors, including prostate cancer. Our purpose in this set of experiments was to determine if HA or HA receptors within the tumor microenvironment could facilitate progression by enhancing the level of 5'-Cap dependent translation within tumor cells. This type of translation has been specifically linked to regulating the expression of genes important in oncogenesis. These genes include cyclin D1, VEGF, and c-myc, and the rationale for testing this hypothesis is due to the linkage between Akt and the downstream effectors important for enhancing 5'Cap dependent translation, such as m-TOR.

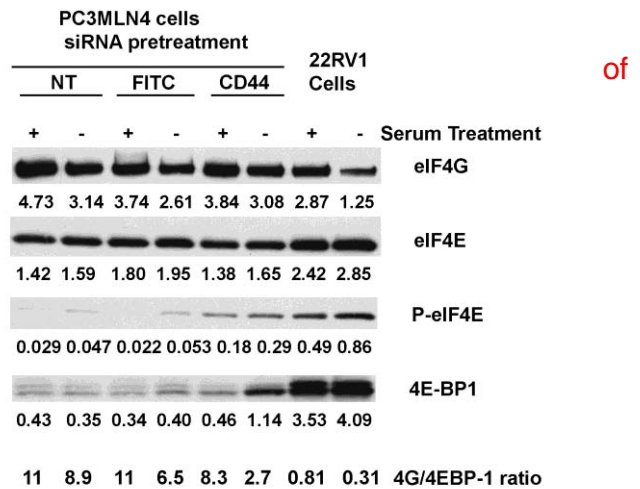
The studies were initiated by first testing the level of 5'Cap dependent translation in prostate tumor cell lines of varying tumorigenic/metastatic potential. The three cell lines were PC3M-LN4 (highly metastatic), 22RV1 (poorly metastatic) and BPH-1 (immortalized cell line from a benign prostatic hyperplasia). The protocol was to examine the relative level of 5-Cap dependent translation in cells that are cultured in the presence (10% serum)_or absence (serum free) of growth/survival factors. Cells were extracted, the contents were purified over an affinity column containing and relative levels of several components of the 5'CAP binding components were quantified.



5'Cap Binding is dependent on eIF4e, which is rate limiting (see Pollunovsky, 2006 for review). The participation of eIF4E in binding the 5'Cap of mRNA is regulated by two components that interact with eIF4e. The first component, eIF4G, facilitates 5' Cap binding of eIF4e to the mRNA. In contrast, eIF4BP, inhibits the binding of eIF4e to the 5'Cap by competing for the binding of eIF4G to the complex. The activity of eIF4BP is regulated by phosphorylation of the protein. Complete phosphorylation of eIF4B at three distinct serine residues inhibits the ability of eIF4BP to bind to eIF4E, leading the constitutive upregulation of 5/Cap dependent translation. The 5'Cap binding assays are performed by affinity chromatography over a 5'guanosyl-coupled resin. To interpret these results the ratio of eIF4G to eIF4BP1 is calculated. A relatively high ratio indicated that 5' Cap dependent translation is active. A relatively low ratio indicates that with the results that 5'Cap dependent translation is constitutively on in the cells. Thus, in the initial experiment the results are as follows.

The three cell types were cultured in the presence (+) or absence (-) of serum and the eIF4E interactions with a 5'Cap affinity column were quantified. The bound components were electrophoresed in SDS-PAGE, transferred for western analysis, and the relative levels of eIF4G and eIF4B were determined. As can be seen, the ratio of 4G to 4BP1 is relatively constant in the metastatic cells in the presence or absence of serum, indicating that the cells can sustain 5'Cap dependent translation in the absence of exogenous survival factors. By contrast, the ratio of 4G to 4BP1 is reduced in the 22RV1 cells, and highly reduced in the BPH1 cells in when serum is depleted in these cultures, indicating that poorly metastatic or immortalized normal prostate cell lines are dependent on serum for robust 5'CAP dependent translation. It is important to note that the steady state levels of these components is evaluated in all experiments prior to affinity chromatography to determine if the various experimental conditions regulate the levels of these components in the cells. In all cases presented, these analyses demonstrated no significant effect on the levels of eIF4G, eIF4E or eIF4BP1 (not shown).

The next experiment performed was designed to evaluate the potential importance of CD44 expression on 5' Cap dependent translation in highly metastatic PC3M-LN4 cells. For these studies, the cells were cultured in the presence or absence of serum for 24 hours as above, and treated with siRNA for CD44, control siRNA FITC labeled, or untreated for an additional 24 hours. Using these conditions, the level of CD44 was inhibited by 80-90% in the CD44 siRNA treated cells. The results are as follows: The 4G to 4BP1 ratios were relatively unaffected in the untreated (NT) cells. By contrast, the ratio of 4G to 4BP1 was reduced 3 fold comparing serum to serum free culture, while the FITC control was reduced 1.5 fold. We are in the process of optimizing the procedure to minimize the nonspecific effects of the FITC negative control siRNA, however these results are consistent with an role for CD44 in facilitating 5'Cap dependent translation. The studies are currently being extended in which we will use QRT-PCR to quantify specific mRNA species with 5'Caps (cyclin D1, VEGF and c-myc) using a mRNA which lacks the 5'Cap (e.g. actin) as control. In the longer term the goal is to define a.) the importance of extracellular hyaluronan in regulating 5'Cap dependent translation in metastatic prostate tumor cells, and to further define some of the signaling pathways involved.



Inhibiting CD44 expression in PC3MLN4 cells decreases translation activity. Cells were pretreated with an siRNA to either CD44 or a control sequence prior to serum starvation for 24 hours. Cell lysate was used in an eIF4E cap binding analog pull down before being run on an SDS-PAGE gel and probed with the indicated antibodies. The translational activity of the cells was measured by the ratio of 4G to 4E-BP1 found in the pull down as determined by densitometry. Untreated and control treated cells were highly translationally active. Cells in which CD44 has been knocked down had reduced activity. Additionally cells lacking CD44 have an increase in phosphorylated eIF4E associated with the cap binding pull down.

Task 3: Use ITC with recombinant proteins to characterize binding of hyaluronan oligosaccharides to CD44 and RHAMM to determine if there is a different size requirement for each receptor. Oligosaccharide size will be based on results of task 2.

Time-Line: years 1 and 2

Milestones and Outcomes: Quantification of binding constants (KDa) of different size HA oligosaccharides to recombinant CD44 and RHAMM protein.

Methods: Isothermal titration calorimetry (ITC) (Pfeil W. and Privalov P.L. Biophys Chem. 1976; 4: 33-40). For this study, HA fragments of specific sizes will be provided by Seikagaku. A buffered solution of hyaluronan fragments will be repeatedly injected (28x10 ul injection, 300-400 sec of spacing) to a buffered solution of recombinant CD44 or RHAMM protein and the energy released as a result of binding between hyaluronan and protein will be measured. Quantification of the released energy will allow calculation of binding constants (KDa).

These studies were started using a different analytical method than the one originally proposed. These studies will utilize surface Plasmon resonance to evaluate the ability of these various synthetic peptides to inhibit HA binding. The studies have only been started in collaboration with the Institute for Therapeutic Drug Delivery at the University of Minnesota. Our collaborator, Dr. Derek Hook, is well qualified to establish this type of analysis. The assay will consist of quantifying the binding of soluble hyaluronan fragments to immobilized recombinant CD44 or RHAMM. The more immediate goal is to determine if these two receptors for HA have different size preferences for HA oligomers. The second longer term will be to define the ability of synthetic peptides with HA binding properties to inhibit HA binding to the two receptors. Again, success in these studies will contribute to the development of specific inhibitors of HA/tumor cell interactions, and will serve as the basis for developing therapeutic small molecule inhibitors.

Task 4: Prepare 1st year annual progress report. Done

Aim 2: *To identify structural features of CD44 and RHAMM that mediate the effects of hyaluronan on signaling pathways regulating tumor growth, survival and invasion*

Task 1: *Generate mutations of CD44 and RHAMM to interfere with hyaluronan-mediated stimulation of tumor growth, survival and invasion*

Time-Line: Years 1 and 2

Milestones and Outcomes: *Site specific mutated constructs of CD44 and RHAMM will be expressed and characterized for the ability to act in a dominant negative fashion to inhibit PC3M-LN4 growth, motility and invasion. Cells that lack or express low levels of CD44 and RHAMM (22Rv1 cells) will be transfected with these mutants to determine if specific mutations fail to mediate hyaluronan stimulated growth or activation of ERK 1, 2.*

Methods: *We have identified key carboxyl amino acids in murine RHAMM by site-directed mutagenesis that are required for interaction with ERK 1/2 (Zhang et al, 1998) and that are conserved in human RHAMM. We will site mutate full length human RHAMM using the approach we developed for the murine mutant (Yang et al., 1994, EMBO J) and tag the mutated cDNA with a FLAG or HA tag (Zhang et al., 1998). The mutated RHAMM cDNA will be stably expressed in PC3LMLN4 cells. We expect this mutated RHAMM form to act as a dominant negative mutant for RHAMM mediated ERK 1/2 ERK activation by homo-dimerizing with endogenous RHAMM expressed by this cell line, as it does in murine fibroblastic tumors. The effect of the expressed mutated RHAMM on an endogenous RHAMM/ERK co-association will be assessed by co-immunoprecipitating RHAMM/ERK complexes using anti-ERK and conversely anti-RHAMM or anti-tag antibodies. The consequences of expressing mutated RHAMM on ERK activity will be assessed using anti-phospho-ERK antibodies in western analysis of proteins separated from cell lysates using SDS-PAGE (Toelg et al., 2005). A putative docking and phosphoacceptor site at Ser of full length human RHAMM has been identified in Blast programs for conserved sequences. We have obtained preliminary evidence that full length RHAMM is phosphorylated in vitro by ERK 2 kinase. We will site mutate Ser15 to Ala and prepare recombinant RHAMM protein for use as a substrate in an in vitro ERK kinase assay and compare phosphorylation with non-mutated recombinant RHAMM protein. If we verify our preliminary results, we will then stably transfect the 15Ser-Ala mutated RHAMM into both PCLMLN4's, to assess effects as a dominant negative regulator of RHAMM function and 22Rv1 cells to assess growth/tumor promoting effects of this mutated RHAMM form. The consequences of this mutated RHAMM on endogenous ERK activity will be assessed as above. Once these analyses are completed, all stable transfectants produced in the Turley laboratory will be shipped to the McCarthy laboratory for further growth/apoptosis assays.*

In Progress. RHAMM expression at high levels by conventional expression vectors is deleterious to cells, requiring the development of alternative expression systems. We have therefore adopted an expression system termed the Rheoswitch inducible expression system, available from New England Biolabs. This system will allow us to precisely induce RHAMM at varying levels of expression in prostate cancer cell lines. The Rheoswitch system consists of two plasmids, the first containing a synthetic nuclear receptor composed of two proteins RheoReceptor-1 and RheoActivator. These two proteins dimerize to form a holoreceptor that blocks transcription of the target gene (in this case RHAMM) in the absence of the synthetic RSL1 ligand. RHAMM will be cloned into the second plasmid and gene expression is controlled by a five tandem repeat of the GAL4 response element. Gene expression is induced by the addition of the synthetic ligand RSL1. RSL1 can be

added at a range of concentrations to induce varying levels of gene expression. Cells expressing RheoReceptor-1/RheoActivator are being generated and clones will be selected for those cells that contain high levels of this vector. Selected clones will then be used as recipients for the expression vector containing RHAMM and the cells will be characterized for RHAMM expression in the presence of different levels of the synthetic RSL1 ligand. These cells will be used in migration/growth and invasion assays *in vitro* and in the longer term they will be used in tumor formation assays *in vivo*, since information from New England Biolabs indicates that this expression system can also be controlled *in vivo* by systemic administration of the ligand.

RHAMM variants with the site specific mutations described above are being developed in the Turley laboratory and should be done in the next month 6 weeks. The constructs will then be shipped to the McCarthy laboratory for expression in prostate cancer cell lines that lack RHAMM expression (e.g. 22RV1 cells, shown above), using the Rheoswitch system described above.

We have expanded the scope of this aim to determine the ability of CD44 to facilitate 5'Cap dependent translation in prostate cancer cells as described above. The implications of these studies are that the tumor microenvironment may enhance 5'Cap dependent translation in malignant tumors, and in so doing contribute to the longer term survival and growth of these cells.

Task 2: Use epistatic approaches to verify the importance of pErk 1/2 in HA mediated tumor cell growth and motility

Time Line: Years 1 and 2

Outcomes and Milestones: Stable cell lines expressing constitutively active or dominant negative MEK1 will be generated and characterized for activation of pERK 1/2, anchorage independent growth and HA mediated motility and invasion.

Methods: Conditional expression vectors will be utilized as described above in Task 1/Aim 1. Stable cell lines will be evaluated for activation of ERK 1/2 and for inhibition of anchorage independent growth/tumor formation in mice. Conditionally expressed dominant negative and dominant active MEK1 cell lines will be shipped to the Turley laboratory and the motile response of these cells to HA and defined sizes of HA fragments will be assessed using both Time-lapse cinemicrography (Toelg et al., 2005) and Boyden-style invasion assays using Matrigel.

These studies are not yet started since the RHAMM expression studies have taken priority.

Task 3: (Year 2). Prepare 2nd year annual progress report. Done

Aim 3: To test the ability of specific hyaluronan-binding synthetic peptides to inhibit HA binding to CD44 and RHAMM or tumor growth/survival *in vitro* and *in vivo*.

Task 1: Identify which residues of synthetic peptide 15-1 are important for binding HA.

Time-Line: year 2

Milestones and Outcomes: Identification of amino acid residues responsible for HA binding.

Methods: Screening of a peptide library using biotinylated hyaluronan as probe resulted in the isolation of a 15 mer peptide with partial homology to a 9 amino acid basic motif (BX7B motif) in the hyaluronan binding region of RHAMM. Based on this information we will synthesize peptides in which basic amino acids have been replaced by, for instance, alanine and the binding constant for binding to hyaluronan will be determined by ITC as described under specific aim 1, task 3.

We have established a collaboration with Drs. Mark Klein and Kevin Mayo (University of Minnesota, Dept. Biochemistry, Biophysics and Molecular Biology) who are NMR spectroscopists. These individuals specialize in determining structural/functional properties of peptides and proteins and Dr. Mayo has long standing interest in peptidomimetics/small molecules that inhibit angiogenesis (Dings et al., 2003; Dings et al., 2005; Mayo et al.,

2003). Peptide 15-1 (p15-1) is a 15 amino acid peptide [STMMRSRSHKTRSHHV] that inhibits HA/receptor interactions. This peptide sequence in the context of its parent protein contains a BX₇B HA binding motif (where B are basic residues and X can be anything except acidic residues) first described in RHAMM, that is also found in many other HA binding proteins (Toole, 2004; Turley et al., 2002). Within the context of its parent peptide, the protein sequence most likely predicts an α -helix motif. However, when the peptide binds HA outside the context of its parent protein, the structural aspects of this interaction are not known with certainty. Structural studies utilizing nuclear magnetic resonance (NMR) techniques are underway to determine the key structural elements in the binding of p15-1 to hyaluronan. NMR can be used to either directly determine a three-dimensional structural model of a peptide, or it can be used to gather evidence for populations of specific structures of that peptide (e.g. α -helix, β -sheet, random coil).

Dr. Klein has recently generated a series of alanine walkthrough variants of peptide p15-1 and we are testing them for the ability to inhibit prostate tumor cell migration. We will use these results in combination with the studies outlined below to determine key residues within peptide 15-1 that are important for HA binding and inhibiting tumor cell migration. Our goal is to use this information for selecting appropriate small molecule libraries to screen for identifying lead compounds. This approach will be performed in parallel with the screening of the calixarene library summarized below. These studies, which are potentially translation in nature, will clearly extend beyond the funding period of the current grant, however studies funded by this grant will be essential for making progress in this area.

The NMR techniques total correlational spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy were used to analyze p15-1 in water solution. A combination of TOCSY and NOESY spectra were used to make the initial amino acid assignments. Analysis of the NOESY peaks did not reveal a significant population of peaks corresponding to alpha-helix or beta-sheet (interactions of specific amino residue atoms < 5 Å apart). Subsequently, p15-1 was placed into solution in the presence of sodium dodecyl sulfate (SDS) or dodecylphosphocholine (DPC) to form micelles. It has been shown that micelles can help to stabilize a peptide that contains interconverting structures. Similar experiments did not yield significant NOESY peaks that correspond to α -helix or β -sheet. However, other NMR techniques have revealed evidence of alpha-helical structure of p15-1 in micelles. Determination of the temperature-dependent changes in chemical shift reveals evidence for hydrogen bonding (and therefore, evidence for alpha-helix in this small peptide) if the ratio of the change in chemical shift relative to the change in temperature ($\Delta\delta/\Delta T$) is more positive than -4.5 parts per billion. Data obtained so far have been at two to three points (5, 20, and/or 40 °C) For p15-1 in SDS, $\Delta\delta/\Delta T = -3.6$ ppb, -3.9 ppb, -2.5 ppb, and -3.4 ppb for residues M4, S5, R6, and H14, respectively. Likewise, for p15-1 in DPC, $\Delta\delta/\Delta T = -4$, -3.9 , and -3.8 ppb for residues S5, S7, and H14. In addition, peptide structure can definitely change upon ligand binding and NMR experiments examining the effect of an hyaluronan octamer binding on the conformation of p15-1 are underway. Any structural differences between the bound and unbound forms of p15-1 would affect the drug design described below.

As part of a collaboration with Dr. Kevin Mayo (U of MN, Biochemistry Dept), we also showed that two α -helix topomimetic compounds based on a calix[4]arene scaffold inhibits the anchorage independent growth of a metastatic hormone independent prostate cancer (cell line PC3M-LN4) at low (micromole) concentrations. This was determined via a screen of a library of these compounds obtained from the Mayo lab. Identifying active compounds in this library would give us several advantages of the use of synthetic peptide, including a lower expense for synthesis and resistance to proteolysis. Calix [4] arenes consist of a cage of 4 benzene rings connected in by intermediary methylene groups (shown in figure below). The diameter of this molecule approximates the diameter of an α -helix or β -sheet, and functional groups can be added to the top or the bottom of the ring to mimic side chains of peptides. The current library of synthesized calixarenes includes 27 variants. The screen referred to above involved 5 representative molecules. Two compounds showed significant inhibitory activity at micromolar concentrations at 7 and 14 days after the start of incubation. Both compounds contain a *tert*-butyl group on each of the benzyl groups on one side of the ring. One of the compounds contains a primary amine connected to each benzyl of the calixarene ring via an ether linkage, and the other consists of a tertiary amine connected to each benzyl via an ester moiety. The other three compounds exhibiting inferior activity differed significantly with respect to the functional groups. These preliminary results suggest different functional groups may be responsible for different calixarene activity in the bioassay. These compounds are currently being analyzed for the ability to inhibit hyaluronan binding to recombinant RHAMM or CD44.

To aid in the design of additional calixarene molecules and to correlate structure and function in an expedited way, we have had 15 peptides synthesized with single site alanine substitutions to determine which residues are important for the binding to hyaluronan in an ELISA assay. A decrease in the binding of a substituted peptide through the use of alanine scanning allows the determination of which residues increase or decrease the binding ability of the peptide relative to the native sequence. The combination of structural and functional properties can be combined to determine the pharmacophore responsible for all or part of a peptide's or small molecule's fundamental activity. The resulting pharmacophore(s) can be overlaid on the calixarene models to design new molecules that mimic the peptide, but are likely to be resistant to proteolysis. Other substitutions can be made that potentially could increase the binding of the peptide relative to the native protein for the design of similar molecules.

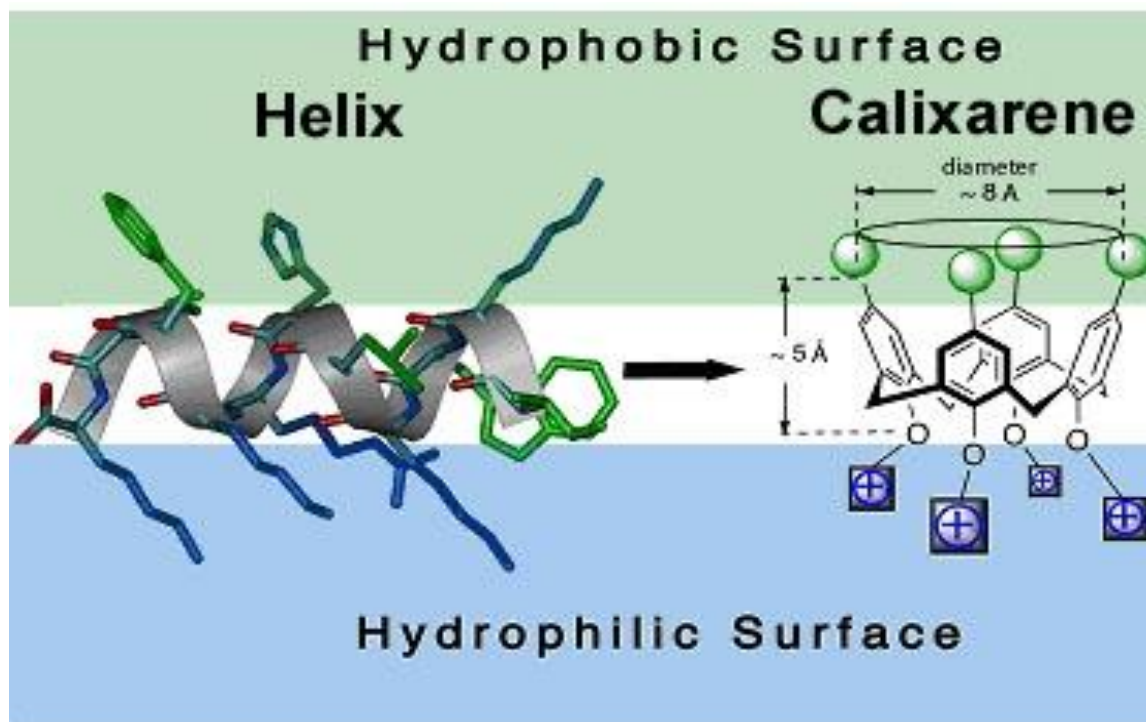


Figure 9. Calixarene scaffold with hydrophobic groups (top) and hydrophilic groups (bottom) superimposed on α -helix

Time-Line: Year 2.

Milestones and Outcomes: Complete studies to determine effectiveness of peptide 15-1 to compete for binding to RHAMM or CD44.

Methods: Peptide 15-1 will be used in competition assays using ELISA type assays of hyaluronan binding to immobilized recombinant RHAMM or CD44.

These studies have not yet been started. The emphasis has first been placed on structural/functional studies described above.

Task 3: *Determine efficacy of peptide 15-1 for inhibiting growth, motility and invasion of prostate carcinoma cells in vitro*

Time-Line: Years 2 and 3.

Milestones and Outcomes: Complete the in vitro analyses of the inhibitory effects of peptide 15-1 in anchorage independent growth, motility and invasion in vitro.

Methods: Peptide 15-1 or appropriate control peptides will be mixed with PC3M-LN4 cells and tested for the ability to inhibit growth in methyl cellulose. Cells pretreated with peptide will also be tested for the ability to activate pERK 1/2 in response to serum, growth factors or hyaluronan. Motility/invasion will be analyzed using both Time-lapse cinemicrography (Toelg et al., 2005) and Boyden-style invasion assays using Matrigel.

These studies have not yet been started. The emphasis has first been placed on structural/functional studies described above.

Task 4: *Evaluate peptide 15-1 for inhibiting tumor growth in orthotopic and intrafemoral injection xenograft injection models for human prostate cancer.*

Time-Line: Years 2 and 3.

Milestones and Outcomes: Complete analysis of inhibitory effect of peptide 15-1 on tumor growth in tumors injected via an orthotopic or intrafemoral route

Methods: Cells will be mixed with peptide and injected into animals either orthotopically or intrafemorally. At the conclusion of the assay, the animals will be sacrificed, the tumors excised from the prostate and weighed/sectioned for histological analysis. Animals in which tumors have been injected into bone (or sham injected) will be X-rayed to estimate bone density and animals will be sacrificed, bones will be harvested and processed for histology. Histological analysis includes estimation of vascularization and hyaluronan content using specific probes available in the McCarthy laboratory.

These studies have not yet been started. The emphasis has first been placed on structural/functional studies described above.

Task 5: (Year 3) Prepare 3rd year annual progress report. Completed.

Task 6 (Year 4) No cost extension final progress report (Completed).

Key Research Accomplishments:

1. Demonstration that cell surface RHAMM has functional significance in wound microenvironments in vivo.
2. Cell surface expression of RHAMM causes sustained activation of the ERK 1, 2 pathway.
3. RHAMM stimulated motility requires CD44 expression
4. Demonstration that cell surface RHAMM forms complexes with CD44 in tumor cells which interact with elements of the ERK 1, 2 pathway.
5. Demonstration that hyaluronan stimulated motility of prostate tumor cells depends on the size of the HA oligomers. Smaller hyaluronan oligomers are more effective than larger oligomers.
6. Demonstration that RHAMM selectively interacts with smaller hyaluronan oligomers, whereas CD44 binds to larger polymers.
7. Generated antibodies against specific HAS isoforms and RHAMM to be used to further evaluate the model proposed above. The antibodies are currently being characterized for reactivity and specificity and will be used in studies to be done during the upcoming year.
8. Generated specific monoclonal antibodies against recombinant Rhamm and demonstrated specific binding of these antibodies in IHC of human prostate specimens. These antibodies are currently being used in combination with anti-CD44 antibodies to evaluate the potential prognostic value of both receptors (alone or in combination) The studies are being performed on tissue microarrays of a well defined cohort of patients which have undergone biochemical recurrence of the disease. These are being done with our collaborator, Dr. Steve Schmechel, a surgical pathologist at the U of MN.
9. Started studies to determine if cell surface CD44 facilitates 5'Cap dependent translation in malignant prostate tumor cell lines.

Reportable Outcomes Articles published during the funding period of this proposal:

Hamilton, S. R., S. F. Fard, et al. (2007). "The hyaluronan receptors CD44 and Rhamm (CD168) form complexes with ERK1,2 that sustain high basal motility in breast cancer cells." J Biol Chem **282**(22): 16667-80.

Maxwell, C. A., J. McCarthy, et al. (2008). "Cell-surface and mitotic-spindle RHAMM: moonlighting or dual oncogenic functions?" J Cell Sci **121**(Pt 7): 925-32.

Tolg, C., S. R. Hamilton, et al. (2006). "Rhamm-/- fibroblasts are defective in CD44-mediated ERK1,2 mitogenic signaling, leading to defective skin wound repair." J Cell Biol **175**(6): 1017-28.

McCarthy, J.B. and E.A. Turley. 2009. RHAMM/HMMR: An Itinerant and Multifunctional Hyaluronan Binding Protein that Modifies CD44 Signaling and Mitotic Spindle Formation Seminars in Cancer Biology.

Conclusion:

Progress has been made on this funded proposal to identify mechanisms by which tumor associated hyaluronan can facilitate tumor growth and invasion. Analysis of human tissue microarrays shows a clear association of increased levels of RHAMM, hyaluronan and hyaluronidase expression. Our studies from two other systems (included in Appendix) have clarified a role for extracellular RHAMM in mediating motility and activation of the ERK 1, 2 pathway both in vivo and in vitro. Furthermore, these studies have demonstrated that RHAMM acts a co-receptor for CD44 in invasive/metastatic breast cancer tumor cell lines and these studies serve as a model to be tested in advanced prostate cancer cell lines used for this study. Furthermore, fragmented hyaluronan was shown to be more effective than higher molecular weight oligomers at binding RHAMM and stimulating prostate tumor cell motility. These studies are consistent with a model in which fragmented HA and RHAMM interact in more advanced tumor cells to stimulate motility and invasion. Finally, a synthetic hyaluronan binding peptide, which was shown in preliminary data in the proposal to inhibit tumor formation in vivo and anchorage independent growth in vivo, has been partially characterized using NMR. The results suggest that the peptide has an interconverting α -helical structure and additional studies in the presence of hyaluronan are being initiated to determine if the interaction stabilizes the conformation of the protein. The goal of these studies is to determine the active residues within the peptide that are important for hyaluronan binding, with the hope that such information could provide a rationale for selecting a small molecule library to further interrogate. Studies using a small molecule library which has properties that would be predicted to be important for binding to hyaluronan have also been started as a parallel approach for designing compounds that will inhibit hyaluronan/tumor cell interactions and reduce growth, motility, and possibly resistance to therapies currently in use.

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