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14. ABSTRACT Despite advances in the diagnosis and treatment of prostate cancer, the inability to inhibit metastasis represents the major cause of failure in the successful treatment of prostate cancer patients. Hyaluronan (HA), a polymeric anionic polysaccharide, is elevated within primary prostate tumors and in the circulation of prostate cancer patients, and this increase is prognostic of poor outcome. Preliminary data demonstrate that HA mediates its effects on tumor cell growth and survival by interacting with two distinct cellular receptors for HA, CD44 and Rhamm (CD168). CD44 and Rhamm share common functional characteristics, such as an ability to bind HA and to activate the Erk/MAPK signal transduction pathway, which is associated with prostate tumor progression. We hypothesize that the HA receptor Rhamm can facilitate tumor growth, invasion and metastasis by compensating for CD44 loss/reduction, particularly when the amount of HA ligand is increased, as is found in high-grade tumors. Determining the mechanisms by which pericellular HA stimulates prostate tumor growth and metastasis may lead to better therapeutics that can be used to limit the disease, allowing for the better clinical management of patients with advanced prostate cancer.					
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Introduction:

Hyaluronan and Control of Normal and Transformed Cellular Growth and Invasion: Hyaluronan (HA) is a large anionic polymeric carbohydrate that influences tissue form and function on the basis of both mechanical and biological properties[1-4]. HA is important for maintaining tissue hydration, cushioning joints and preserving cell free space within specific tissues. During development, HA is required for many morphogenetic events such as neural crest cell migration, cardiac development and ductal branching of the prostate gland. HA is also an important adhesion/migration substrate during wound healing and elevations in HA are associated with epithelial to mesenchymal transitions during development[5]. Hyaluronan is synthesized in mammals by one or more members of a family of three hyaluronan synthases (HAS1, 2, or 3).[6-8] Newly synthesized HA may be deposited into HA-rich matrices, or alternatively degraded and internalized. Degradation of HA occurs by the concerted action of both exoglycosidases that sequentially remove carbohydrates from the reducing end of the polymers and endoglycosidases (known as hyaluronidases) that cleave HA polymers into relatively large oligosaccharides[1] These fragments may be internalized and degraded further or stimulate angiogenesis if released to the extracellular environs. Polymeric HA is secreted as a free polymeric carbohydrate, however cellular receptors for HA, along with specific extracellular HA-binding proteins and proteoglycans, bind HA to retain and organize it within the extracellular milieu[9, 10].

Hyaluronan-rich matrices alter the growth and survival of both normal and transformed cells. Hyaluronan interactions with specific cell associated hyaladherins (CD44 and Rhamm, see below) are associated with enhanced responsiveness to growth and survival factors[2]. Gene array analysis of synchronized cells indicates that transcripts for both HAS2 and the HA receptor Rhamm (see below) are increased at G2/M [11] and HA synthesis increases at this stage of the cell cycle.[1, 12, 13] Small (2500 dalton) HA oligosaccharides, or truncated constructs of cellular HA receptors that disrupt the interaction of polymeric HA with receptors on tumor cells, inhibit the anchorage-independent growth and/or invasion of tumor cells *in vitro* and tumor formation *in vivo*. [14-16]. Elevated levels of HA within the primary tumor are an independent negative prognostic indicator in prostate cancer (see below), suggesting that HA can enhance progression-associated malignant properties of tumors.

Primary prostate tumor progression is accompanied by significant increases in both hyaluronan deposition and hyaluronidase levels in the tumor-associated stroma and in the carcinoma, respectively.[17-19] Perineural infiltration, seminal vesicle invasion by tumors and PSA recurrence are associated with the intensity of stromal HA staining in prostate cancer patients undergoing radical prostatectomy[20]. The interplay of hyaluronan synthases and hyaluronidases results in the formation of HA rich matrices with heterogeneous-sized polymers and fragments of HA that can also facilitate tumor angiogenesis[17, 18]. Retrospective analysis of human prostate (and other) tumor specimens has shown that the ratio of hyaluronidase:hyaluronan expression is an independent prognostic indicator, consistent with the hypothesis that fragmented hyaluronan in the tumor microenvironment is associated with the malignant potential of the tumor.[21, 22] As prostate tumors progress to become metastatic, or acquire androgen independence following therapy, carcinomas may develop the ability to synthesize their own HA by multiple mechanisms[23]. Pericellular HA is essential for prostate tumor growth and enhanced angiogenesis in a xenograft subcutaneous injection model [24] and in orthotopic or intrafemoral bone injection models (see progress report below). Importantly, the addition of highly purified hyaluronan (1 mg/ml) to the injection medium of cells inhibited from synthesizing their own HA, by antisense-mediated removal of HAS expression from these cells, completely reverses growth inhibition[24].

CD44 and Rhamm – Two Hyaladherins with Overlapping Function: The two most studied HA receptors include CD44 and Rhamm (Receptor for Hyaluronan Mediated Motility). Both of these receptors are implicated in tumor progression and metastasis, and they have both been shown to affect tumor adhesion, invasion, growth and survival.[2, 25, 26] Both of these proteins are structurally unrelated and are encoded by unique genes located on distinct chromosomes (11p13 and 5q33, respectively). Despite their unique structure, they appear to have overlapping (but not identical) functions based on their ability to bind HA, regulate HA mediated motility and invasion, and regulate growth factor/survival factor pathways in both normal and transformed cells.

CD44 is an integral type I transmembrane HA binding protein that is highly polymorphic due to extensive alternative splicing and post-translational modifications.[25, 27] Structural variation in CD44 expression occurs as a result of alternative splicing of the gene. CD44 is also extensively glycosylated at both N and O-linked sites on the core protein. Not all CD44 isoforms bind HA, although the mechanisms that govern this functional variability are not completely understood. N-linked glycosylation and the presence/absence of a cytoplasmic tail have both been implicated in regulating HA binding. The CD44 core protein can also be expressed as a chondroitin sulfate or heparan sulfate cell surface proteoglycan, and this type of modification has been associated with influencing binding to other ECM ligands (fibronectin, collagen), binding/presenting specific growth factors and enhancing activation of their cognate signaling receptors.[16, 28] While some studies indicate that increased CD44 expressed is a bad prognostic factor for certain cancers (e.g. renal cell cancer, papillary thyroid carcinoma and breast cancer), other cancers (e.g. head and neck, prostate cancer and ovarian cancer) appear to be characterized by decreased CD44 expression.[27] CD44 can be a positive effector in tumor invasion and metastasis, whereas in other tumors (e.g. prostate, see below) it has been implicated as a metastasis suppressor[2, 25, 27, 29, 30]. While the exact reasons underlying these discrepancies are not understood, it is clear that contradictions can arise from a number of factors, including the differences in the antibodies used, and the genetic and epigenetic background of the cells being used. **Expression of endogenous or transfected CD44 in human prostate cancer cell lines promotes anchorage independent growth *in vitro* (see below), suggesting that CD44 may be important for tumor cell survival/growth early in progression, when CD44 is still expressed at moderate to high levels.**

While both clinical and laboratory data overwhelmingly implicate a positive role for tumor-associated HA in prostate tumor progression, paradoxically the data also indicate that more advanced prostate tumors are also characterized by a decrease or loss in CD44 expression. In multiple independent histopathological studies, decreased levels/intensity of CD44 staining was associated with progression in primary prostate tumors.[31-34] The decrease in CD44 expression in patient samples is due to methylation of the promoter for CD44, suggesting that the local microenvironment could modulate CD44 expression.[33, 35] This may help to explain apparently contradictory observations documenting increased CD44 levels in prostate tumor cells that have entered the circulation.[36] CD44 is also associated with the internalization of HA, which is important for hyaluronidase-mediated degradation of this high molecular weight polysaccharide.[37, 38] Not surprisingly, loss of CD44 expression in inflammation models using CD44 $-/-$ mice show enhanced accumulation of HA in the afflicted tissues with a corresponding increase in the severity of the inflammatory response.[39] By analogy, decreased levels of CD44 associated with more advanced prostate cancers could further exacerbate the enhanced formation of pericellular HA-rich matrices. The resulting increased level of HA could stimulate prostate tumor growth by interacting with a functionally overlapping HA receptor (e.g. Rhamm).

Rhamm is another major hyaladherin that, like CD44, is important for HA-mediated motility and growth. Rhamm is expressed as both an intracellular and cell surface (CD168) protein.[25, 40-44] The mechanisms by which Rhamm associates with the cell surface are not known, since the molecule lacks both a conventional signal sequence and a putative transmembrane domain. Rhamm is one example of an increasing number of proteins that can be secreted or released by non-conventional mechanisms and that have unique functions depending on their subcellular distribution (reviewed in).[45] Cell surface Rhamm (CD168) has been implicated in promoting the motility/invasion of multiple cell types, based on the ability of specific anti-Rhamm antibodies to inhibit these responses. Rhamm ligation by HA has also been associated with the activation of intracellular signaling molecules associated with growth and survival such as ras, c-src, focal adhesion kinase (FAK) and mitogen activated protein (MAP) kinases.[25, 46, 47] Rhamm $-/-$ mice exhibit severe defects in excisional wound repair and in HA/PDGF stimulated fibroblast migration ([48]). This migratory defect is correlated to defects in the activation of the MAP kinase/Erk pathway, which can be partially reversed by the stable expression of constitutively active MEK1 ([48]). Inhibiting Rhamm expression or function in ras-transformed fibroblasts or mouse embryonic fibroblasts blocks transit through G2/M by inhibiting the level of cdc2/cyclin B1, which is essential for mitosis.[49] Intracellular Rhamm (also known as Intracellular HA Binding Protein, or IHABP) associates with both the actin cytoskeleton and with microtubules, where it has been proposed to function in the assembly of intracellular signaling complexes/pathways associated with cell growth[25, 26, 43], and intracellular hyaluronan and/or Rhamm have been co-localized with elements of the

mitotic spindle [2, 13, 25, 26, 40, 43]. Cell cycle analysis of fibroblasts isolated from Rhamm $-/-$ mice have an exaggerated percentage of cells in G2/M (Hamilton and Turley, manuscript in preparation), further supporting this conclusion.

Multiple isoforms of Rhamm have been detected in both normal and transformed cells. In human breast cells, one full length transcript (Rhamm_{fl}) and three major splice variants have been described[43] one lacking exon 4 (Rhamm Δ exon4), a second lacking exon 13 (Rhamm Δ exon13), and a third splice variant that lacks both exons 4 and 13(Rhamm Δ exon4,13). While the biological significance of these splice variants is not well understood, studies have shown that subcellular distribution of Rhamm is altered by the presence, or absence of exon 4. Rhamm_{fl} has been shown to associate with interphase microtubules and the mitotic spindle, whereas Rhamm Δ exon4 does not associate with interphase microtubules[43]. Interestingly, in contrast to Rhamm_{fl}, Rhamm Δ exon4 concentrates within cell nuclei, suggesting that it may have nuclear targets that influence chromatin organization or gene expression. It has also been shown that the ratio of Rhamm Δ exon4/Rhamm_{fl} increases with disease severity in myeloma patients.[41] Variants that lack exon 13 are generally present in much smaller amounts than either Rhamm_{fl} or Rhamm Δ exon4, and no biological significance has yet been attached to the loss of exon 13. However, intracellular injection of anti-Rhamm antibodies that bind to the carboxyl terminal region of Rhamm disrupts mitotic integrity, leading to abnormal polarities in the mitotic spindle.[40]

Rhamm expression is elevated in a number of tumors, including carcinoma of the breast, bladder, stomach, tumors of neural origin, and hematopoietic malignancies.[50-53] More recently, Rhamm expression has been shown to be important for the initiation and formation of aggressive fibromatosis (also known as desmoid tumor) in an animal model for this tumor.[54] While Rhamm seems important for the initiation of mesenchymal tumors, the data from patient samples suggest Rhamm overexpression/redistribution may occur later in carcinoma progression. Late stage carcinomas partially replicate aspects of developmental processes involving epithelial to mesenchymal transition, suggesting that Rhamm expression may be one aspect of a fibroblast signature in tumors, which has been associated with aggressive malignancies. In humans, infiltrating breast carcinomas contain elevated levels of Rhamm/IHABP in the trabeculae and at the invasive edges of the tumor, implicating Rhamm overexpression in promoting invasion and metastasis[26]. Furthermore, in certain tumors Rhamm may compensate for decreased levels of CD44 in HA-rich tumor microenvironments. While progression of transitional cell carcinoma of the bladder is associated with decreased levels of CD44 expression, Rhamm expression was positively correlated with progression[51].

It has recently been shown that Rhamm can replace CD44 as an HA receptor in promoting collagen induced arthritis.[39] The inflamed joints of the knockout mice showed enhanced accumulation of HA compared to wild type joints, and removal of HA in either genetic background inhibits the joint inflammation induced by collagen injection. The increased accumulation of HA within the inflamed tissues of CD44 $-/-$ mice is not surprising, given the role of CD44 in promoting HA clearance from tissues.[38] Antibodies against Rhamm (or recombinant Rhamm injected into the tissues) inhibited inflammation in the joints of CD44 $-/-$ mice, indicating that Rhamm serves as an HA receptor that can mediate inflammation in the absence of CD44[39]. This concept is particularly novel, in that functional overlap/molecular redundancy in this model is between two hyaluronan receptors that have radically distinct structures, subcellular distribution and HA binding sites. Importantly, although both receptors bind HA and impact upon cell motility and growth, the joints of inflamed CD44 $-/-$ mice are more severely impacted compared to the joints of CD44 wild type mice. HA mediated motility of leukocytes from CD44 $-/-$ mice (which could be blocked by anti-Rhamm antibodies) was significantly greater compared to that exhibited by wild type mice. This suggests that although Rhamm can functionally overlap with CD44, it does not function identically to CD44 in the context of joint inflammation. The reasons for this difference between the function of CD44 and Rhamm are not yet clear. The function of Rhamm as both a cell surface and intracellular protein that targets the cytoskeleton and the nucleus suggests that some of the differences may be due to alterations in Rhamm-mediated gene expression in the presence or absence of CD44. Consistent with this hypothesis are gene array data from the two knockout animals indicating that the number of transcripts altered by inflammation was more than 3 times as great in the CD44 $-/-$ mice compared to wild type mice, and 18 of these transcripts were directly or indirectly related to inflammatory

processes.[39] By analogy, Rhamm expression in later stage carcinomas, which exhibit decreased CD44 expression, could result in aggravated HA-mediated tumor cell growth, survival, and invasion.

Decreases in Rhamm transcript expression are associated with activin-induced apoptosis of LNCaP tumor cells *in vitro*, consistent with a role for Rhamm in facilitating prostate tumors growth and survival[55-57]. Furthermore, a recent genome wide scan of a cohort of brothers with prostate cancer has linked several chromosomal regions (5q, 7q, and 19q) with more aggressive disease (based on comparative Gleason scores).[58] The linked region in chromosome 5q (5q31-33) includes the Rhamm locus, although Rhamm expression *per se* was not evaluated in these studies. While the region of chromosome 5q linked to aggressive prostate cancer disease is admittedly much larger than the Rhamm locus, the results are consistent with the possible importance of Rhamm in late stage malignant progression. **Our working model is that the HA matrix synthesized and assembled by metastatic prostate cells creates a microenvironment that facilitates tumor cell survival, growth and invasion. The hypothesis is that Rhamm can partially compensate for loss of CD44 function in advanced tumors in the presence of elevated levels of hyaluronan. We propose that selective interference with HA/tumor cell interactions is a viable therapeutic approach for inhibiting tumor cell growth and survival *in vivo*.**

Body:

Statement of Work:

Aim 1: To evaluate the ability of HA 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..

Time-Line: year 1

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 siRNA's and by stable transfection with conditional expression vectors coding for specific shRNA's. 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 FACS™.

CD44 and Rhamm specific antibodies and siRNA's 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.

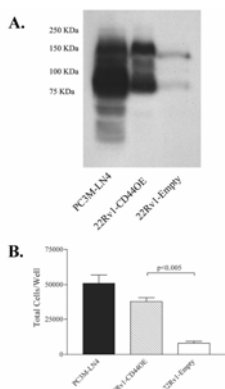


Figure 1. Over-expression of CD44 increases the anchorage independent growth potential of the 22RV1 CaP cell line. Western Analysis of transient over-expression of standard CD44 in 22RV1 cell line (A). Twenty-four hours after transfection cells were harvested and 15,000 were seeded into methylcellulose. Seven days later the growth assay was harvested and cells counted using trypan blue (B).

The studies in this aim are in progress, and have been modified. Studies are in progress to develop specific shRNAs that will inhibit Rhamm or CD44 expression. Cells that are stably transfected will be selected using drug markers and flow cytometry. The other approach is to stably express constructs of either CD44 or Rhamm in prostate cancer cells that lack each receptor. We have identified a human cell line (22RV1) that expresses low or nondetectable levels of both receptors. The cells have been stably transfected with a vector expressing the standard isoform of CD44 or full length Rhamm and each population is at various stages of *in vitro* characterization (Figures 1 and 2). CD44 transfectants were isolated by drug selection and flow cytometry. The cell population expressed levels of CD44 similar to prostate cancer cells (e.g. PC3M-LN4) that express endogenous CD44 (not shown). These cells have been characterized for the ability to grow in an anchorage independent fashion (Figure 1). The results show that CD44 expression facilitates anchorage independent growth to a level

that is equal to PC3M-LN4 cells, whereas mock transfectants grow poorly under these conditions. Furthermore, we are generating stable transfectants that express epitope tagged full length Rhamm

(Figure 2). These cells are currently under drug selection for the purpose of isolating stable transfectants. These cells will also be tested for the ability to grow/migrate in vitro and in vivo.

Furthermore, we have had to generate additional antibodies against Rhamm for the purpose of using these antibodies in vitro to inhibit cell motility/growth and for the purpose of evaluating expression of this receptor in cell lines and within tumor sections. This has taken more time than anticipated, since Rhamm antibodies have proven problematic in the past. We have selected three regions of the protein from which to generate synthetic peptide immunogens. These come from the amino terminal third, the central, and the carboxyl terminal regions of the protein (not shown). Antibodies were produced in Rabbits, the sera were collected and the Ig fraction purified. The antibodies generated against each region of the peptide were used in western blot, flow cytometry and for staining tissue sections. Results using anti-Rhamm

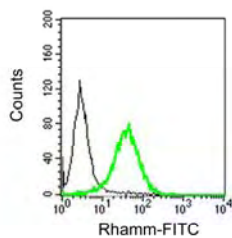


Figure 3. Flow Cytometry analysis of PC3 CaP cells stained with polyclonal Rhamm antibody. The black line represents background staining with secondary alone. Rhamm staining is represented by the green line.

antibodies generated against the central region of the protein are shown. The antibodies stained the cell surface of PC3M-LN4 cells (Figure 3) and they were used to effectively stain prostate cancer samples on tissue arrays (Figure 4). The results show that Rhamm is not expressed in normal prostate or benign prostatic hyperplasia, but that it is expressed in advanced prostate cancer specimens,. This is in contrast to CD44, which is expressed in normal and BPH specimens, but is not detected in malignant lesions of the prostate (Figure 5). All polyclonal antibodies are being tested for the ability to inhibit HA mediated motility and growth.

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

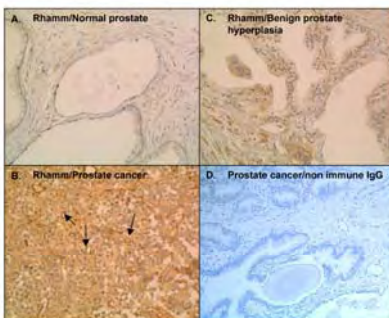


Figure 4. Rhamm is expressed in benign prostate hyperplasia and prostate cancer but not in normal prostate tissue. Tissue arrays of normal prostate (A.), benign prostate hyperplasia (C.), and prostate cancer (B.) were stained with anti-Rhamm antibodies. Non-immune IgG served as a control for non-specific staining (D.). Rhamm is detected in the luminal epithelium, stroma, and blood vessels of cancer (arrows) and hyperplasia.

either CD44 or Rhamm (Figure 6B). This experiment demonstrates that expression of one or both receptors is important for stimulating motility in response to HA. Furthermore, the results indicate that aggressive metastatic prostate tumor cells respond best to smaller oligomers of HA, consistent with a model in which these fragments (which would be enriched in more

In Progress. We have used a wound healing motility assay to evaluate the ability of various sizes of HA fragments to migrate (Figure 3). HA oligomers of different molecular weights were added to wounded cultures of PC3M-LN4 cells and the ability of these oligomers to stimulate wound closure was evaluated (Figure 6). The motility of PC3M-LN4 cells (which express both Rhamm and CD44) was stimulated by the smallest oligomer tested (9 kD, Figure 6A), whereas oligomers larger than that either had no effect or slightly inhibited motility. In contrast, these same oligomers had no effect on stimulating the motility of parental 22RV1 cells, which express low/nondetectable levels of

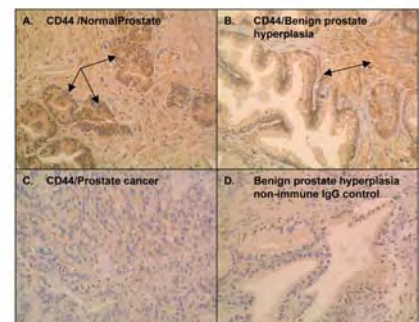


Figure 5. CD44 is expressed in normal and benign hyperplasia of prostate tissue but is reduced or lost in anaplastic prostate cancer. Tissue arrays of normal prostate (A.), benign prostate hyperplasia (B.), and prostate cancer (C.) were stained with anti-CD44 antibodies (KM101). Non-immune IgG served as a control for non-specific staining (D.). CD44 protein is detected in the luminal epithelium and stroma of normal and hyperplastic prostate tissue (arrows) but is much reduced or not detected in prostate cancer.

advanced tumors) could act to facilitate invasion and metastasis. The experiments are being repeated with additional sizes of HA. The prediction is that smaller sizes of oligomers will be stimulatory for motility, however very small oligomers (in the range of 2500 kD) may cause cells to under apoptosis. Furthermore, stable transfectants of 22RV1 cells expressing only CD44 or Rhamm will be tested in both motility and anchorage independent growth assays. The prediction is that Rhamm expressing cells will be more motile, whereas CD44 expressing cells will be more responsive in terms of growth. Additionally, FACE analysis will be performed for tumors grown in xenografts to validate the presence of the various size HA oligomers within the tumor. Experiments are also in progress to evaluate the effect of hyaluronidase treatment of tumor cells on the growth and metastasis in orthotopic injection xenograft models. The prediction is that hyaluronidase will enhance tumor invasion and metastasis in this model system.

Time-Line: year 1

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.

Task 3: Use ITC with recombinant proteins to characterize binding of HA 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.

This task has not yet been started.

Time-Line: years 1 and 2

Milestones and Outcomes: Quantification of binding constants (Kd) 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 HA 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 HA and protein will be measured. Quantification of the released energy will allow calculation of binding constants (Kd).

Not Started

Task 4: Prepare 1st year annual progress report.

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

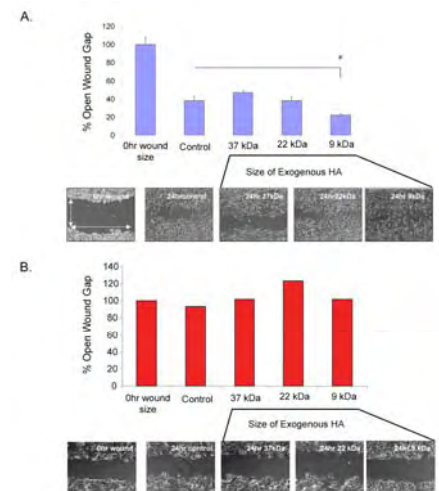


Figure 6. Effect of different HA fragment sizes on wound gap closure by human CaP cells. Migration of serum starved PC3M-LN4 cells (A) or ZR75.1 (B) into gaps of scratch wounds. Monolayers were measured in the presence of 37 kDa, 22 kDa, or 9 kDa HA (LifeCore). Wound closure was measured as the size 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 ZR75.1.

Task 1: Generate mutations of CD44 and Rhamm to interfere with HA-mediated stimulation of tumor growth, survival and invasion

This task is not yet started.

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 HA 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 Ser¹⁵ 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.

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

This task is not yet started.

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 Timelapse cinemicrography (Toelg et al., 2005) and Boyden-style invasion assays using Matrigel.

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

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 HA 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 HA 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 HA will be determined by ITC as described under specific aim 1, task 3.

Task 2: Evaluate peptide 15-1 for inhibiting HA binding by CD44 and Rhamm

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 HA binding to immobilized recombinant Rhamm or CD44.

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 HA. Motility/invasion will be analyzed. using both Timelapse cinemicrography (Toelg et al., 2005) and Boyden-style invasion assays using Matrigel.

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 HA content using specific probes available in the McCarthy laboratory.

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

Key Accomplishments:

Year 1

1. Stable expression of CD44 promotes anchorage independent growth in vitro
2. Full length Rhamm has been expressed in human prostate cancer cells
3. New antibodies generated against Rhamm recognize cell surface and intracellular isoforms.
4. IHC of human tissue samples show that Rhamm and CD44 have reciprocal expression profiles in normal prostate and malignant tumors.
5. Small fragments of HA stimulate the motility of metastatic prostate tumor cells while large fragments are not stimulatory
6. Cells that lack CD44 or Rhamm do not migrate in response to HA oligomers

Reportable Outcomes:

Year 1

1. Rhamm and CD44 expression is reciprocal in prostate tumor specimens
2. Small HA oligomers, such as would be anticipated to be in advance prostate tumors, stimulate motility of metastatic prostate cancer cells
3. Rhamm or CD44 expression is important for HA mediated motility, since cells that lack either receptor fail to migrate in response to HA oligomers

Conclusions:

Year 1:

The results are consistent with a model in which CD44 and Rhamm can both mediate tumor cell growth and motility in response to HA. The motility of these cells is stimulated by small HA oligomers, consistent with a role for HA degradation/tumor invasion in more advanced tumors, as has been demonstrated previously by others. During the upcoming year, the cell lines generated will be used to test the importance of Rhamm vs. CD44 in stimulating motility and growth as well as being important for preferential response to specific sizes of HA fragments.

Abbreviations:

Akt	serine/threonine kinase important for cell survival
DU145	Androgen Independent Human Prostate Tumor Cell Line
eGFP	enhanced Green Fluorescent Protein
Erk 1/2	serine kinase pathway component important for stimulating growth, survival and motility
FACE	Fluorophore-assisted Carbohydrate Electrophoresis
FAK	Focal Adhesion Kinase-important in cell adhesion and motility
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HA	Hyaluronan
HAS	Hyaluronan Synthase
HYG	hygromycin resistance gene
IRES-eGFP	Expression vector with internal ribosomal entry site allowing simultaneous expression of gene of interest plus eGFP
IRES-HYG	Expression vector with internal ribosomal entry site allowing simultaneous expression of gene of interest plus HYG
LNCaP	Androgen Responsive Human Prostate Tumor Cell Line
pAkt	Phosphorylated Akt
pErk 1/2	Phosphorylated (active) version of erk1/2
PC3	Androgen Independent Human Prostate Tumor Cell Line
PC-3M	Metastatic Variant of the PC3 Human Prostate Tumor Cell Line
PC-3MLN4	Lymph Node Metastatic Variant of the PC3M Human Prostate Tumor Cell Line
PI3K	Phosphatidylinositol 3 kinase-has many functions including activation of Akt
PTEN	Tumor suppressor (Phosphatase and <u>T</u> ensin Homologue on Chromosome 10)
shRNA	small hairpin interfering RNA
siRNA	small interfering RNA
Rhamm	Receptor for Hyaluronan Mediated Motility (CD168)
RT-PCR	Reverse Transcriptase Followed by Polymerase Chain Reaction
22Rv1	Androgen Independent Human Prostate Tumor Cell Line

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