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14. ABSTRACT
Despite advances in the diagnosis and treatment of prostate cancer in the last several years, metastasis represents the major cause of frustration and failure in the successful treatment of prostate cancer patients. Hyaluronan (HA) is polymeric anionic carbohydrate that is elevated within primary prostate tumors, most notably within the tumor-associated stroma. We have demonstrated that increased HA synthesis by human prostate carcinoma cells correlates with metastatic potential. This increased synthesis results from the elevated expression of specific hyaluronan synthases (HAS) in the tumor cells. Metastatic prostate carcinoma cells exhibiting high levels of HAS assemble and retain a pericellular HA matrix on their cell surfaces. We have used vectors to stably express constructs encoding antisense for HAS enzymes to study the importance of elevated hyaluronan synthesis in prostate carcinoma adhesion, growth and tumor formation. The studies outlined in this annual report document our observations that support an important role for hyaluronan in prostate tumor progression. metastasis.

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INTRODUCTION

Prostate cancer is a major cause of cancer-related deaths in men. [1] While most prostate cancer is confined to the prostate at the time of diagnosis, patients with carcinomas that progress to malignancy will eventually harbor tumors that are increasingly invasive and vascularized. Malignant progression ultimately culminates in various degrees of visceral invasion and metastasis to lymph nodes and bone. Metastasis to bone is especially noteworthy, not only because it reflects more advanced tumors, but also because of the intense debilitating bone pain that often accompanies bone metastases. Newly diagnosed tumors may be treated by one of several methods including androgen ablation, however clinical complications arise when tumors become androgen-independent and resume growth. By defining factors that contribute to the growth and metastasis of androgen-independent tumors, it may be possible to better diagnose and treat prostate cancers by inhibiting growth of primary tumors or metastases. This would allow for better clinical management and enhanced quality of life for prostate cancer patients.

Hyaluronan (HA) is a large anionic polymeric carbohydrate that influences tissue form and function on the basis of both mechanical and biological properties. [2-5] HA-rich matrices are found in several normal adult tissues, including vitreous, cartilage, and the central nervous system. 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. [6]

Hyaluronan is synthesized in mammals by one or more members of a family of three hyaluronan synthases (HAS). [7-9] The loci encoding the three HAS isozymes are located on three separate chromosomes [*HAS1* (19q13.3-q13.4), *HAS2* (8q24.12) and *HAS3* (16q22.1)]. Structural predictions of the three isozymes suggest that each contains 6 membrane-spanning domains along with a seventh membrane associated domain. [7] The active site and substrate binding domains are located on a large intracellular loop. All three isozymes catalyze the formation of the HA-polymeric repeating disaccharide motif by utilizing alternating UDP-D-glucuronic acid and UDP-N-acetyl-D-glucosamine donors. The molecular weight of polymers varies from 10^5 to 10^7 daltons. Polymerization is concurrent with extracellular secretion, and the evidence to date is that HA synthesis by cells is regulated by transcription of specific HAS isoforms. [7]

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. These may be internalized and degraded further where they may modify cell growth intracellularly or stimulate angiogenesis if released to the extracellular environs. [10] While HA is secreted as a free glycosaminoglycan, its incorporation into extracellular matrices that contain HA-binding proteoglycans and link proteins serves to facilitate HA retention within these matrices. [11, 12] Link proteins and proteoglycans bind to HA using a common structural motif known as a link homology domain. [11, 12] These loosely organized matrices can modulate the diffusion of nutrients and small molecule effectors, and several growth factors/cytokines have been identified that may bind directly to specific components within HA-rich matrices, becoming locally concentrated as a result of these interactions.

Primary prostate tumor progression is accompanied by significant increases in both hyaluronan deposition and hyaluronidase levels in the tumor-associated stroma. [13-15] This HA-rich matrix is also populated by newly forming blood vessels that are produced as part of the angiogenic response to the tumor. [14, 15] The interplay of hyaluronan synthases and hyaluronidases results in the formation of matrices with heterogeneous-sized polymers and fragments of HA. HA polymers can cluster and aggregate cell surface HA receptors such as CD44. [4] Alternatively, CD44 may help to promote HA fragment internalization [16]leading to further degradation of small HA oligomers that may localize to the cytoplasmic matrix where they are available to interact with other HA receptors such as RHAMM/IHABP or elements of the mitotic spindle. [17-19] Released HA fragments may also contribute to tumor-induced angiogenesis. [14] This heterogeneous

mixture of HA polymers and oligosaccharides may therefore stimulate multiple pathways important for tumor cell growth, survival and metastasis by interacting with various HA receptors expressed by the carcinomas.

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. Segments of chromosome 8q.24, which are overrepresented in prostate cancer, contain the coding sequences of several genes that are upregulated in the tumor, including c-myc and HAS2. [20] This suggests that increased HA synthesis in prostate cancer may result in part from an underlying genetic defect. Soluble factors within prostate tumors may also contribute to upregulation of HAS isozymes and HA synthesis in the tumor. [21-24] Decreased CD44 in the tumors could also contribute to decreased internalization of HA with a concurrent upregulated deposition in the tumor. These factors, along with the action of specific HA receptors on the tumor cells could enhance growth/invasion of the tumor, increase angiogenesis, enhance metastasis to lymph nodes or facilitate growth within the bone marrow microenvironment at sites of metastasis.

Our preliminary data for this proposal indicated that upregulated HA synthesis by metastatic prostate carcinoma cells enhanced their adhesion to bone marrow endothelial cell lines. Furthermore, we demonstrated that elevated hyaluronan synthesis in these cells was caused by the upregulation of two mammalian hyaluronan synthases (HAS 2 and HAS 3). The HA synthesized by the metastastic prostate carcinomas was retained on the surface of these cells as a pericellular matrix, that can be visualized microscopically by a red cell particle exclusion assay (see articles in Appendix). The major hypothesis to be tested in the proposal is that disruption of HA synthesis by metastatic tumor cells will limit tumor growth, vascularization/angiogenesis, and will limit metastasis to lymph nodes and bone.

Body

STATEMENT OF WORK

Specific Aim #1: Determine the role Of HA biosynthesis in prostate tumor cell growth and invasion.

Months 1- 12: Subcutaneous and intraprostatic injections of malt athymic nude mice with stable cell lines (PC3M-LN4) expressing antisense constructs for appropriate HAS isoforms.

The subcutaneous injection experiments have been finished and published (Simpson et al., Am. J. Path. 2002). The results show that HA synthesis is important for prostate carcinoma growth both in vitro and in vivo. We have also correlated HA synthesis by metastatic prostate tumor cells to propensity to adhere to bone marrow derived endothelial cells in vitro. These results are published in Simpson, et al., J. Biol Chem. 2002. Collectively, the results indicate that upregulated hyaluronan synthase expression in metastatic prostate tumors leads to the formation of a pericellular hyaluronan matrix around the tumor cells. These matrices appear to be important for mediating the adhesion and growth (both in vitro and in vivo) of the tumor cells. Studies are continuing using more relevant sites for injection, such as the prostate capsule and direct injection of tumor cells into the bone marrow.

Months 3-12: Analysis of tumor tissue from above injections by RT-PC, antibody staining HA detection, and histology.

The tumor tissues have been analyzed using hematoxilin and eosin to visualize tumor cells and overall architecture of the tumors. We have also estimated tumor associated hyaluronan levels using a specific biotinylated hyaluronan binding protein that we have isolated from bovine nasal cartilage. Tissue associated hyaluronan is visualized using strep-avidin peroxidase and diaminobenzidine. Using this approach, we have demonstrated that tumor associated hyaluronan is reduced in tumors formed by cells stably expressing antisense constructs for hyaluronan synthase 2 or 3, either alone or in combination. Furthermore, we have evaluated angiogenesis within frozen sections of tumors by using anti-CD31 antibody and immunofluorescence. The images were digitized and analyzed for average staining intensity (quanitified as average pixel density). The results clearly demonstrated that angiogenesis of prostate carcinomas correlates to HA synthesis by the tumors. The results are published in Simpson, et al. Am. J. Pathol. 2002.

Months 4-12, In vitro characterization of stable cell lines (LNCaP) overexpressing HAS isoforms.

We have performed studies to evaluate HAS expression by LNCaP cells. We chose these cells originally because of their poor metastatic potential. Our working model is that highly metastatic cells prostate tumor cells may acquire the ability to metastasize in part because of their autonomous production of hyaluronan and their assembly of this hyaluronan into a pericellular matrix. Although these cells do not express hyaluronan synthases, they also have no detectable levels of CD44 or another HA receptor that has become a recent subject of interest in our studies. This receptor, termed RHAMM, is upregulated in more metastatic prostate tumor cells compared to poorly metastatic counterparts. As a result, the simple addition of HA to LNCaP cells has no detectable effect on stimulating cell growth in vitro. Furthermore, LNCaP transfectants expressing HAS isozymes show no detectable increase in

growth despite a high level of HA synthesis brought about by transfection of the active HAS isozymes.

Months 6-12; injection of characterized LNCaP cell lines in mice to test for tumorigenic potential

We have had technical difficulty generating stable transfectants of LNCaP cells. As a result, we have examined other cell lines for HAS expression and/or HA receptor expression...We have shown that 22RV1 cells, which are androgen independent tumor that grew out of the androgen dependent transplantable CWR22 tumor, expresses very low levels of CD44 and another HA receptor termed RHAMM. We are now using these cells to overexpress HAS enzymes and to compare the relative importance of RHAMM and CD44 overexpression in facilitating HA rich matrix formation and tumor growth/vascularization.

Month 12: Prepare annual progress report

Completed and Submitted 2.04

Specific Aim #2: Characterize the effect of altered HA levels on prostate carcinoma metastasis.

Months 6-12: Evaluation of spontaneous metastases resulting from intraprostatic, injection of PC3MLN4 cell lines.

This study is in progress. We have performed initial studies to develop the model in our laboratory. We have successfully been able to reproducibly obtain tumors following injection of parental PC3M-LN4 cells into the prostatic capsule. The cells form large metastases in the regional lymph nodes. Experiments are in progress to determine the importance of upregulated HA synthesis in tumor growth and metastasis.

Our results demonstrate the following:

Orthotopic injection models for prostate cancer have been used to demonstrate the importance of the microenvironment in dictating tumor growth, invasion and metastasis. Orthotopic injection of PC3M-LN4 cells into the mouse prostate yields robust tumors within 4-6 weeks following injection. These tumors also metastasize aggressively to lymph nodes, with 70-80% of the animals injected with parental or mock-transfected cells having visually detected regional lymph node metastasis. Inhibiting HA synthesis in tumor cells also inhibits growth within the prostate and lymph node metastasis is reduced to less than 10%. Based on additional odds-ratio statistical analysis of these data (performed by the University of Minnesota Cancer Center Biostatistics Core), we conclude that lymph node metastasis in this model correlates to tumor size, which is in turn related to HA synthesis of the tumor. The addition of exogenous HA to cells prior to injection reversed the inhibitory effects of the antisense construct, as was observed in subcutaneous tumorigenicity studies.

We have also adopted a direct bone injection model to evaluate the importance of tumor-associated HA in tumor growth within the bone marrow microenvironment. While the model does not take into account the steps necessary for tumor metastasis to bone, it does provide a useful system for evaluating factors that are important for regulating tumor growth and vascularization in the bone marrow microenvironment. This model has been used to evaluate tumor growth, tumor-induced bone resorption, and tumor-induced bone pain by our consultant Dr. Denis Clohisy. The bones from the mice (8/group) were fixed, demineralized and processed for histocytochemistry. The percentage of animals in which bone tumors were detected following intrafemoral injection with either parental PC3M-LN4 or mock-

transfected control tumor cells ranged from 50-75%. Femurs injected with mock- transfected or parental tumor cells (not shown) contained significant areas of tumor growth. Morphometric estimates of the bone marrow area occupied by the expanding parental or mock-transfected tumors revealed that 20-45% of the marrow space was overtaken by the prostate tumor. The tumors are lytic in nature, causing fractures in a percentage of the tumor-bearing animals. Importantly, visual examination of the femurs injected with antisense expressing tumor cells revealed no evidence of tumors in any of the animals. Staining for hyaluronan in growing mock-transfected tumors reveals a well organized HA matrix within the bone lesion, with limited amounts of HA detected in the bone marrow microenvironment using these fixation conditions. The results indicate that tumor-associated HA is important for stimulating tumor growth in multiple tissue environments, and that HA is particularly critical for tumor cell survival/growth within the bone marrow microenvironment.

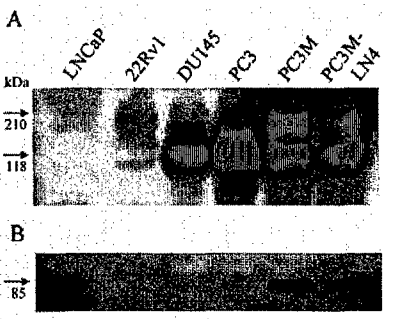


Figure 4. Western analysis of CD44 and RHAMM for prostate cell lines. Lysates from six prostate cancer cell lines were obtained run on a 12% SDS-PAGE then transferred to a nitrocellulose membrane. Membranes were blocked and probed with a 1:750 dilution of mouse hCD44H-(C25) (A) or 1:500 dilution of rabbit anti-hRHAMM R3.7 (B). Proteins were detected with 1:50,000dilutions of horseradish peroxidase-conjugated anti-mouseor anti-rabbit antibody.

Hyaluronan could impact tumor growth *in vivo* by multiple effects on both the tumor and the tumor associated stroma. **We hypothesized that tumor-associated hyaluronan acts directly on tumor cells in an autocrine fashion to stimulate tumor growth.** To test this hypothesis, we adapted a methylcellulose assay to evaluate anchorage independent growth of prostate carcinoma cells *in vitro*. The cells growing in this assay form large multicellular colonies, similar to what is observed in agarose. This assay offers advantages over agarose since the gels can be easily solubilized, allowing for recovery and quantification/biochemical characterization of cells at the end of the experiment. After 7 days of incubation, the cells were recovered from the gels and counted. The results show that PC3M-LN4 cells and mock-transfectants plated at low density (30 X 10^3/culture) within these matrices exhibit anchorage-independent growth over the 7 days of the assay. Inhibiting HA synthesis using HAS antisense vectors causes a significant (75-80%) inhibition of growth that can be reversed by the addition of highly purified hyaluronan (LifeCore, Chaska, MN). Reversal of growth inhibition was most notable in the presence of higher molecular weight HA (220 and 800 kD). The results are similar to what we observed *in vivo* following subcutaneous injection of tumors, leading to the conclusion that pericellular HA matrices synthesized by the tumor have a direct autocrine effect on stimulating metastatic prostate tumor growth *in vitro* and *in vivo*.

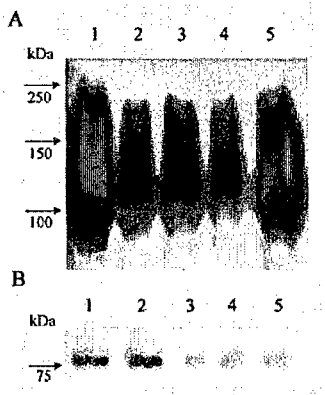
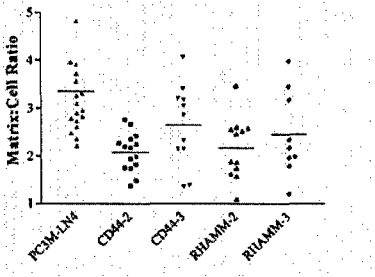


Figure 5. Western blot analysis for CD44s and RHAMM siRNA treated cells. PC3M-LN4 cells were treated with 20 μM of respective siRNA's for 48 h. The negative siRNA control (Lane 1) was ran as a control for both gels. Cells treated with siRNA's for CD44s were run in Lanes 2-5 (A) and siRNA's for RHAMM were run in Lanes 2-5 (B). Lysates were collected and ran in 7.5% SDS-PAGE then transferred to nitrocellulose membranes. Membranes were blocked and probed with a 1:750 dilution of mouse anti-hCD44H-(C25) (A) or 1:500 dilution of rabbit anti-hRHAMM R3.7 (B). Proteins were detected with 1:50,000 dilutions of horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody.

We next evaluated the expression of HA receptors in prostate carcinoma cells, which have varying degrees of tumorigenic/metastatic potential. Initial RT-PCR resulted in multiple sized amplicons for CD44 and only a single size amplicon for RHAMM (not shown). The expression of several other possible HA receptors (i.e. Layilin, LYVE-1, TLR-4) was also evaluated by RT-PCR but these have not yet been detected. Western blots of prostate cancer cell lysates were probed for CD44 and RHAMM. Poorly metastatic LNCaP cells express no detectable CD44 and minimal levels of RHAMM. 22Rv1 cells, which are androgen nonresponsive cells generated from parental androgen responsive CWR22 cell line express low levels of CD44 splice variants and also contain barely detectable levels of RHAMM. Metastatic DU145 and PC3 cells express easily detectable CD44 (primarily migrating at 118 kD with minor higher bands) and they also express easily detectable levels of a single species of RHAMM



	CD44-2	CD44-3	RHAMM-2	RHAMM-3
PC3M-LN4	p<0.0001	p=0.0424	p=0.0004	p=0.0189

Figure 6. CaP exhibit decreased matrix following transfection with siRNA. Following 48 hours of siRNA treatment PC3M-LN4 cells were plated for particle exclusion assay. Student t-tests were performed on pairwise comparisons of interest.

that migrates at approximately 85 kD (which is the same apparent molecular weight as full length RHAMM). PC3M and PC3M-LN4 cells (which were generated as metastatic variants from the PC3 cell line) exhibit even further increases in RHAMM expression (with equal or slightly decreasing levels of CD44 compared to the PC3 line). Studies using exon-specific antibodies are in progress to further define the exact nature of the CD44 variants. We are continuing (with the assistance of our collaborators Drs. Turley and Savani) to determine if other RHAMM variants might be present at lower levels, as has been shown for other human tumors. **However, we conclude that human prostate tumor cell lines with increasing metastatic potential are characterized by increased expression of full length RHAMM.**

Months 13-20, Intraprostatic injection of LNCaP cell lines and evaluation of tumorigenicity and metastasis.

We have abandoned these studies due to a technical difficulty in obtaining stable transfectants of LNCaP cells. We are in the process of preparing stable transfectants of 22RV1 cells in which we will express HAS2, HAS3 or RHAMM and/or CD44 in these cells. Our goal is to develop a model system in which we can evaluate the specific role of an HA pericellular matrix in promoting tumor cell growth via interaction with specific tumor cell associated HA receptors. We have initiated stable transfections as of March of 2004. We anticipate having stable transfectant in 2-3 months for testing.

Months 13-20: Standardize conditions for intracardiac Injection of prostate cancer cells and analysis of metastases.

Depressing HA synthesis inhibits growth and vascularization of tumors in a number of microenvironments. As a result, we have abandoned the cardiac injection assay (to model bone metasasis) and instead we have adopted a direct bone injection assay to model tumor growth in that micronenvironment. We will use that injection model as one of our biological readouts for developing the 22Rv1 cell line model system

Months 16-24: Perform metastasis studies using HAS variant prostate carcinoma cells in intracardiac injection model.

We have abandoned the cardiac injection model as described immediately above.

Months 16-24: Extend studies on prostate tumor cell/bone marrow endothelial cells using parallel plate flow assay.

These assays are also on hold. They are in vitro correlates of tumor cell arrest and extravasation, however our focus has changed from metastasis per se to growth/survival in specific microenvironments. As a result, these studies as originally proposed are no longer a priority.

Month 24- Prepare second annual report and submit new application

Completed and Submitted 04.04

Specific Aim #3: Examine the impact of HA on tumor cell colonization of bone marrow in a mouse model and in bone marrow stromal cell co-culture

We have extended these studies as described above using a direct intrafemoral injection assay and have published the results in a meeting proceeding (HA 2003, Article in Appendix). . The results demonstrate that inhibition of HA synthesis inhibits tumor growth following direct intrafemoral injection. The studies are summarize and results shown in the appended article.

Months 20-30. Standardize conditions for direct bone injection and analysis of prostate carcinoma cells

Done

Months 24-36: Examine the impact of tumor HA synthesis on tumor growth/expansion within bone.

These studies have been extended from the first year and the results included in the Appended article.

Months 22-26: Evaluate effects of prostate tumor cell/bone marrow stromal cell co-culture on tumor growth *in vitro*

These studies were abandoned to pursue the importance of prostate tumor HA receptor expression in tumor growth. The results using cell lines show that the expression of Rhamm, an HA receptor important for motility and growth, is increased in more aggressive cell lines. The most studied HA receptor, CD44, has been shown to decrease as function of progression in CaP, yet HA levels continue to increase. Both of these phenotypic changes of tumors portend a poor prognosis, suggesting that alternate HA receptors are expressed by more advance prostate tumors. To test this possibility, we first used semi quantitative RT-PCR to screen for additional receptors in CaP cell lines, including Rhamm, LYVE-1, Stabilin 1 and 2. The only HA receptors identified were Rhamm and CD44. Furthermore, using tissue microarrays, we have shown that Rhamm expression in CaP epithelial cells occurs late in progression, in late stage (Gleason 8, 9) that have lost glandular architecture, implicating this receptor in HA mediated tumor metastasis. We have used siRNA technology to inhibit expression of Rhamm or CD44 in these cells, and then tested the effects of this inhibition on anchorage independent growth in vitro. Reduction of either of these HA receptors significantly inhibits tumor cell growth, and this inhibition can be reversed by the addition of excess HA to the cultures. In contrast, when both HA receptors were simultaneously knocked down, the inhibition of growth was almost complete, and exogenous HA could not reverse this inhibition. We conclude that either HA receptor can stimulate CaP growth, and that each receptor may somehow complement the actions of the other receptor. From the standpoint of tumor progression, we further speculate that CD44 may enhance HA mediated growth/survival earlier in progression, whereas more advanced tumors (Gleasons 8, 9) that have lost glandular architecture may utilize Rhamm in invasion and metastasis formation. We have also used siRNA treatment of cells to demonstrate an inhibition of growth following injection of tumor cells in xenograft models. We are now generating cell lines with conditional expression vectors using shRNA to further define the importance of these two receptors in prostate tumor progression. These results served as preliminary data for submission of a DOD Prostate grant for the 2005 award cycle.

Month 36: Prepare annual report

This grant received a no-cost extension at the conclusion of year 3, so this report for Year 3 is an annual report.

Key Research Accomplishments

Year 1

1. Prostate/bone marrow endothelial cell interactions depend on increased production of hyaluronan by the tumor cells.

2. Elevated hyaluronan synthesis results from increased expression of two specific hyaluronan synthases, called hyaluronan synthase 2 and hyaluronan synthase 3

3. Transfection of constructs encoding hyaluronan synthase 2 or 3 results in increased hyaluronan synthesis by poorly tumorigenic cells

4. Poorly tumorigenic cells expressing increased hyaluronan exhibit increased adhesion to bone marrow endothelial cell lines

5. Upregulated hyaluronan synthase expression in metastatic tumor cells can be inhibited by stably transfecting vectors that encode antisense hyaluronan synthase 2 or hyaluronan synthase 3, either alone or in combination

6. The hyaluronan dependent adhesion of metastatic cells to bone marrow endothelial cell lines can be inhibited by using antisense approaches to inhibit expression of hyaluronan synthase 2 and hyaluronan synthase 3, either alone or in combination

7. Subcutaneous tumor growth is inhibited by stable transfection of antisense HAS 2 or HAS 3.

8. Angiogenesis of subcutaneous tumors is greatly (90%) reduced in tumors formed by prostate carcinoma cells in which hyaluronan synthesis has been inhibited.

9. Inhibition of tumor growth or angiogenesis observed in the antisense expressing cells can be reversed by the addition of exogenous hyaluronan at the time of injection

Year 2

10. Demonstrated that HA synthesis is important for intraprostatic growth

11. Related HA synthesis to regional lymph node metastasis in orthotopic tumors

12. Related HA synthesis to growth/survival in bone marrow microenvironment

13. Demonstrated that HA synthesis acts in autocrine fashion for metastatic tumors

14. Obtained preliminary data to demonstrate that HA is an autocrine factor for tumors

15. Obtained preliminary data to demonstrate that RHAMM is upregulated in metastatic prostate tumor cells

Year 3

16. Inhibited RHAMM or CD44 expression and demonstrated both mediate growth via HA

17. Inhibition of expression of both Rhamm and CD44 completely inhibits CaP growth in vitro and this cannot be reversed by HA

18. Rhamm is expressed in late stage CaP specimens obtained from patients

19. Synthetic peptides that disrupt HA/Tumor Cell Interaction have been identified and are being developed as lead compounds for inhibiting CaP growth.

Reportable Outcomes

Year 1

1. Elevated hyaluronan synthesis in human metastatic androgen independent tumor cells leads to the formation of a pericellular matrix rich in hyaluronan.

2. Tumor cells with a pericellular hyaluronan matrix adhere avidly to bone marrow endothelial cell lines

3. Inhibition of the synthesis of this matrix by using antisense constructs to inhibit expression of specific hyaluronan synthases inhibits adhesion to bone marrow endothelial cells, tumor growth *in vitro* and *in vivo*, and tumor induced and angiogenesis in vivo

Year 2

4. Inhibiting HA synthesis decreases anchorage independent growth in vitro which can be reversed with exogenous HA

5. Inhibiting HA receptor expression/function also inhibits HA matrix formation and anchorage independent growth *in vitro*.

Year 3

6. Inhibiting either CD44 or Rhamm inhibits tumor growth that can be reversed by exogenous HA.

7. Inhibiting expression of both receptors simultaneously can almost completely inhibit tumor cell growth in vitro that cannot be reversed by the addition of exogenous HA

8. Inhibiting expression of either receptor inhibits tumor growth *in vivo*

9. Identification of peptide structures based on the HA binding site of Rhamm and CD44. These peptides are being tested for the ability to inhibit growth/survival of CaP both *in vitro* and *in vivo*.

Conclusions

Year 1

We conclude that elevated hyaluronan synthesis in metastatic prostate carcinoma cells is an important factor for stimulating tumor adhesion, growth and angiogenesis. The results suggest that the synthetic apparatus for hyaluronan may be a potential target in advanced prostate tumors. Current efforts are focused on evaluating the importance of elevated hyaluronan in promoting tumor growth within the prostate capsule, metastasis to regional lymph nodes, and growth/angiogenesis within the bone marrow microenvironment.

Year 2

Our working model is that the HA matrix synthesized and assembled by metastatic prostate cells provides the cells with their own microenvironment that facilitates tumor cell adhesion to endothelium, invasion and growth within tissues. Based on results obtained during the last year, we hypothesize that increased RHAMM expression in metastatic prostate cancer cells leads to HA-mediated invasion, anchorage-independent growth and survival *in vitro*, and facilitates tumor formation and metastasis *in vivo*. Current efforts using stable transfections and RNAi are focused on testing this hypothesis directly.

Year3

During this last year of funding, we have further pursued the relationship between HA synthesis, CD44 expression and Rhamm expression in prostate cancer samples. Hyaluronan could impact tumor growth *in vivo* by multiple effects on both the tumor and the tumor associated stroma. **We originally hypothesized that tumor-associated hyaluronan acts directly on tumor cells in an autocrine fashion to stimulate tumor growth.** To test this hypothesis, we adapted a methylcellulose assay to evaluate anchorage independent growth of prostate carcinoma cells *in vitro*. The cells growing in this assay form large multicellular colonies, similar to what is observed in agarose. This assay offers advantages over agarose since the gels can be easily solubilized, allowing for recovery and quantification/biochemical characterization of cells at the end of the experiment. After 7 days of incubation, the cells were recovered from the gels and counted. The results show that PC3M-LN4 cells and mock-transfectants plated at low density (30 X 10^3/culture) within these matrices exhibit anchorage-independent growth over the 7 days of the assay (see appendix article). Inhibiting HA synthesis using HAS antisense vectors causes a significant inhibition of growth that can be reversed by the addition of highly purified hyaluronan (LifeCore, Chaska, MN). Reversal of growth inhibition was most notable in the presence of higher molecular weight HA (220 and 800 kD). The results are similar to what we observed *in vivo* following subcutaneous injection of tumors, leading to the conclusion that pericellular HA matrices synthesized by the tumor have a direct effect on stimulating metastatic prostate tumor growth *in vitro* and *in vivo*. These studies have led to the hypothesis that prostate tumor cells express two HA receptors that can mediate growth. CD44, which is present early in progression, can stimulate growth, motility and invasion in the presence of HA. Rhamm, which is expressed in late stage carcinomas (determined by staining Tissue Microarrays for Rhamm) can also respond to HA resulting in an aggravated malignant behavior of late stage tumor cells, when CD44 is reduced or absent. Continuing studies are in progress to define the nature of the functional overlap between these two HA receptors in the context of tumor growth, survival and motility in vitro and in vivo. Furthermore, in very recent studies, specific peptide structures that bind HA with moderate affinity have been identified and will be used to interfere with tumor cell HA interactions. The peptides will be evaluated for the ability to inhibit HA- stimulated tumor growth, motility and tumorigenic potential when injected orthotopically or intrafemorally into xenograft models.

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*Appendix**

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Final Reports

N/A (This is an annual report)

Appendix for 2005 Annual Report

Hyaluronan Biosynthesis in Prostate Carcinoma Growth and Metastasis

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Keywords: Prostate Cancer, Rhamm, CD44, Bone Metastases, Lymph Node Metastases

Abstract

Elevated levels of hyaluronan are associated with malignant progression of prostate cancer. Progression from the primary tumor is associated with atypical growth and glandular architecture, invasion into the surrounding stroma and metastasis to distant sites, including bone. Treatment options for patients with advanced disease include androgen ablation therapy which fails due to the evolution of androgen-independent tumor cells within patients. We have previously shown that highly metastatic, androgen-independent human prostate tumor cells synthesize high levels of hyaluronan and incorporate this hyaluronan into pericellular matrices *in vitro*. The high level of hyaluronan synthesis by these cells is due in part to increased levels of hyaluronan synthases 2 and 3. By contrast, poorly metastatic or tumorigenic human prostate tumor cell counterparts have low levels of hyaluronan synthases and correspondingly low levels of hyaluronan synthesis. Highly metastatic prostate tumor cells utilize hyaluronan to adhere avidly to bone marrow endothelial cell lines *in vitro*, implicating tumor- associated hyaluronan in homing to bone. Furthermore, hyaluronan synthesis also facilitates the growth and vascularization of metastatic human prostate tumor cells both in vitro and in vivo following subcutaneous injection into immunocompromised mice. Subcutaneous tumor formation of metastatic human prostate cancer cells is inhibited 60-80 percent as a result of antisense-inhibition of hyaluronan synthase expression. Tumor-associated angiogenesis is inhibited to a similar degree, implicating tumor-derived hyaluronan in promoting tumor angiogenesis and vascularization. Importantly, the growth of tumors harboring stable hyaluronan synthase antisense constructs can be completely reversed by the addition of exogenous hyaluronan at the time of injection. These results indicate that it is the product of the hyaluronan synthases, rather than the synthases per se, that are important for facilitating tumor growth. Our current efforts are focused on evaluating the importance of tumor cell associated hyaluronan in facilitating tumor growth and metastasis in more physiologically relevant sites, such as within the prostate gland or the bone microenvironment. The results suggest an important role for hyaluronan in the biology of prostate cancer progression and metastasis.

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Hyaluronan Biosynthesis and Prostate Tumor Growth and Metastasis

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 [1-5]. 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 [6]. Hyaluronan is synthesized in mammals by one or more members of a family of three hyaluronan synthases (HAS1, 2, or 3) [7-9]. 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 can 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 [10, 11].

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, 5, 12]. Gene array analysis of synchronized cells indicates that transcripts for both HAS2 and the HA receptor Rhamm (see below) are increased at G2/M [13] and HA synthesis increases at this stage of the cell cycle [1, 14, 15]. Small (2500 dalton) HA oligosaccharides, or recombinant protein fragments of cellular HA receptors that antagonize HA binding to endogenous cellular receptors, inhibit the anchorage-independent growth and/or invasion of tumor cells *in vitro* and tumor formation *in vivo* [16, 17]. 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 [18-20]. Perineural infiltration, seminal vesicle invasion by tumors and PSA recurrence are all associated with a high intensity of stromal HA staining in prostate cancer patients undergoing radical prostatectomy [21]. 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 [18, 19]. Retrospective analysis of human prostate (and other) tumor specimens has shown that an increased ratio of hyaluronidase:hyaluronan expression is an independent indicator of poor prognosis, consistent with the hypothesis that partially fragmented hyaluronan in the tumor microenvironment is associated with the malignant potential of the tumor [22, 23]. 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 [24]. Pericellular HA is essential for prostate tumor growth and enhanced angiogenesis in a xenograft subcutaneous injection model [25] and in orthotopic or intrafemoral bone injection models (preliminary

results). 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 *in vivo* [25]. These cells also have high levels of hyaluronidase which has been shown to partially cleave high molecular weight hyaluronan into smaller fragments [18]. Based on this observation, and studies linking the level of hyaluronidase activity to prostate cancer progression [22, 23] it is quite likely that partial cleavage of high molecular weight HA by endogenous hyaluronidase creates fragments of hyaluronan that stimulate prostate tumor growth and angiogenesis.

Data presented below support the hypothesis that prostate cancer growth and metastasis is an HA-mediated process. Hyaluronan synthesized by both the tumor associated stroma (in primary tumors) or by the carcinoma cells in more advanced lesions can enhance tumor formation by androgen independent tumor cells. Furthermore, data are presented to demonstrate that HA associated with tumor cells is important for orthotopic growth. regional lymph node metastases and growth within the bone marrow microenvironment. The results suggest that interfering with tumor: HA interactions could be an effective therapeutic strategy in the treatment of patient with advance prostate cancer.

Materials and Methods:

Cell Culture - PC3M-LN4 cells were the kind gift of Dr. I.J. Fidler (M.D. Anderson Hospital Cancer Center, Houston, TX). PC3M-LN4 cell lines were grown in MEM containing 10% fetal bovine serum, sodium pyruvate, and nonessential amino acids. Media supplements and antibiotics were purchased from Invitrogen (San Diego, CA). Stably transected HAS antisense cell lines were generated and maintained as described previously [26].

Three-dimensional Growth - A 2% solution of methylcellulose in dH_2O was sterilized by autoclaving and allowed to stir for 8 hours at RT. The solution was then aliquoted and frozen at –20°C until used. The 2% solution of MC was allowed to thaw overnight at 4°C. Prostate tumor cells were trypsin released, washed and resuspended in 2X growth medium at 15,000 cells/ml. Using an 18 gauge needle, 1 ml of 2% methylcellulose was added to 1 ml of cell suspension and vortexed briefly to mix. The 2 ml cell suspension was added to a sterile non-tissue culture treated 35 mm petri dish, placed in a 100 mm petri dish along with another 35 mm dish that contains sterile dH_2O, and incubated at 37°C/5% CO_2. On the seventh day, pictures were taken using a phase contrast microscope at 10X and 40X. Total cell counts were performed by diluting the colonies formed in the 1% methylcellulose gel with 1X PBS to a total volume of 20 ml and centrifuging at 1,500 rpm for 5 min. Subsequently, the cells were suspended in 350 µl of a 0.05% Trypsin/EDTA solution to break up colonies and incubated at RT for 10 min. Cells were mixed with trypan blue and a total viable cell count performed. As appropriate, HA was added to the cell suspension prior to the addition of methylcellulose. Experiments were performed in triplicate and statistically analyzed by two-tailed Students t-test.

Subcutaneous tumor growth - All animal studies were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota. Prostate adenocarcinoma cells were injected subcutaneously into the flank of male NOD.CB17$^{-Prkdcscid}$ mice (3-5 week old, Jackson Laboratory, Bar Harbor, Maine) using a 28 Ga needle. Tumor growth was monitored for 4-6 weeks post-injection by caliper measurements of length (longest

diameter) and width (perpendicular to length) and volume estimated. Tumor volume (mm^3) was estimated by $V= (4\pi)/3 \bullet r^3$, where r is an estimated radius calculated by r= (length + width)/2. At the completion of the study mice were euthanized and necropsy performed. The subcutaneous tumors were excised and wet weight determined. Subsequently, the tumors were fixed in 10% buffered formalin for later histological and immunohistochemical staining. Differences in tumor weight between control and treated groups were assessed by two-tailed Student's t-test.

Direct bone injection - Based on the procedure described by Clohisy and associates [27, 28]. The femora of anesthetized male NOD.CB17$^{-Prkdcscid}$ mice were injected, after knee arthrotomy, with 20 µl phosphate buffered saline (PBS) or prostate tumor cell suspension using a 30 Ga needle and the wounds closed with surgical staples. Animals were euthanized 28-35 days post injection and the bones were removed, fixed, and de-mineralized. Processed bones were sectioned and stained using hematoxylin/eosin to visualize the bone marrow cavity. Data were expressed as a percentage of injected femurs in each experimental group that contain tumor. Alternatively, the mean percentage area of bone marrow occupied by tumor was estimated using morphometry on 5 random sections of bone.

Orthotopic injection model - Based on the procedure described by Pettaway and associates [29]. Male NOD.CB17$^{-Prkdcscid}$ mice were anesthetized, a lower midline incision is made, and the dorsal prostatic lobes exposed. Using a 30 Ga needle, one dorsal lobe was injected with 20 µl of prostate tumor cell suspension while the contra-lateral lobe was injected with phosphate buffered saline as sham control and the abdominal wound closed. Growth of tumor in the prostate gland was initially assessed by palpation. Animals were euthanized 35-42 days after injection and evaluated for primary tumor growth and lymph node metastases. The tumors were excised, weighed and processed either as frozen tissues or paraformaldehyde fixed tissues. Harvested primary tumors and lymph nodes were processed for histology/immunohistochemistry to evaluate tumor growth and HA content.

Results and Discussion

Hyaluronan Stimulates Prostate Cancer (CaP) Cell Growth *in vivo* and *in vitro*

Our previous studies demonstrated that metastatic prostate carcinoma cells assemble their HA into a pericellular matrix, as has been demonstrated for other cell types [25, 26, 30]. Furthermore, these cells express two of the three mammalian hyaluronan synthases (HAS2 and HAS3). Interfering with the expression of these two isozymes (using HAS antisense vectors to generate stably transfected cells) resulted in inhibition of HA synthesis and pericellular matrix formation by these cells. Adhesion to bone marrow endothelial cells was similarly inhibited. We also demonstrated that inhibiting hyaluronan synthesis in metastatic, androgen independent tumor cells significantly inhibits tumor growth and tumor induced angiogenesis following subcutaneous injection. This inhibition of tumor growth and vascularization could be completely reversed by the addition of exogenous hyaluronan at the time of injection [25]. This indicates that the presence of high levels of hyaluronan in the tumor microenvironment, rather than the carcinoma-associated HAS enzymes *per se*, is critical for tumor cell growth and survival i*n vivo*.

Orthotopic injection models for prostate cancer have been used to demonstrate the importance of the microenvironment in dictating tumor growth, invasion and metastasis [29]. Orthotopic injection of PC3M-LN4 cells into the mouse prostate yields robust tumors within 4-6 weeks following injection ([29] and Figure 1). These tumors also metastasize aggressively to lymph nodes, with 70-80% of the animals injected with parental or mock-transfected cells having visually detected regional lymph node metastasis (not shown). Inhibiting HA synthesis in tumor cells with HAS anti-sense also reduces growth within the prostate (Figure 1) and lymph node metastasis is reduced to less than 10% (not shown). Based on additional odds-ratio statistical analysis of these data (performed by the University of Minnesota Cancer Center Biostatistics Core), we conclude that lymph node metastasis in this model correlates to tumor size, which is in turn related to HA synthesis of the tumor. The addition of exogenous HA to cells at the time of injection reversed the inhibitory effects of the antisense construct, as was observed in subcutaneous tumorigenicity studies [25]. We have also adopted a direct bone injection model to evaluate the importance of tumor-associated HA in tumor growth within the bone marrow microenvironment. While the model does not take into account the steps necessary for tumor metastasis to bone, it does provide a useful system for evaluating factors that are important for regulating tumor growth and vascularization in the bone marrow microenvironment [27, 28, 31]. The bones from the injected mice (8 mice/group) were fixed, demineralized and processed for histocytochemistry (Figure 2). The percentage of animals in which bone tumors were detected following intrafemoral injection with either parental PC3M-LN4 or mock-transfected control tumor cells ranged from 50-75% (not shown). Femurs injected with mock-transfected (Figure 2B) or parental tumor cells (not shown) contained significant areas of tumor growth. Morphometric estimates of the bone marrow area occupied by the expanding parental or mock-transfected tumors revealed that 20-45% of the marrow space was overtaken by the prostate tumor (not shown). The tumors are lytic in nature, causing fractures in a high percentage of the tumor-bearing animals. Importantly, visual examination of the femurs injected with HAS-antisense expressing tumor cells revealed no evidence of tumors in any of the animals

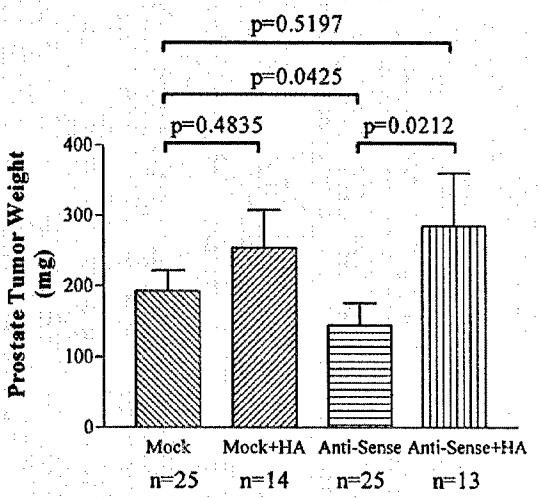

Figure 1. Orthotopic growth of HAS2/3 anti-sense CaP cell lines. Briefly, $1x10^5$ mock or anti-sense transfected PC3M-LN4 cells were injected into the left lobe of the prostate. 28 days later the glands were harvested and weighed. Post-hoc t-tests were conducted on pairwise comparisions to determine statistical significance.

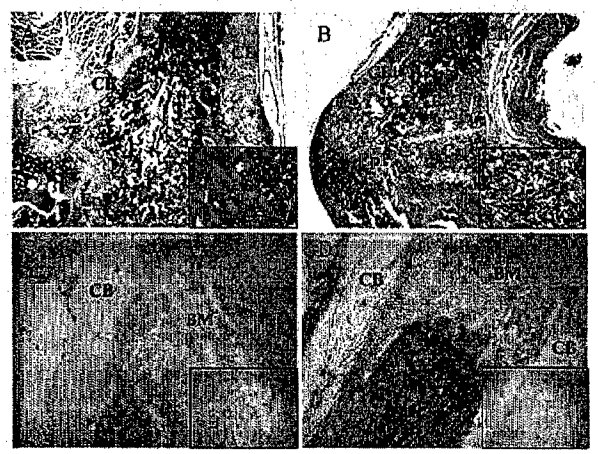

Figure 2. Intrafemoral growth of HAS2/3 anti-sense CaP cell lines. H+E stained sections of femurs injected with mock (B) or anti-sense CaP (A) cells (100X), inset 400X. Mock (D) and anti-sense CaP (C) femurs stained for hyaluronan (100X) using a biotinylated HA binding protein, streptavidin-HRP, and DAB. These sections were not countersained with Mayer's Hematoxylin to more readily visualize HA localization. Insets are hyaluronidase treated conrol sections (100X).
CB-cortical bone, BM-bone marrow, CaP-prostate carcinoma

(example in Figure 2A). Staining for HA in growing mock-transfected tumors reveals a well organized HA matrix within the bone lesion, with limited amounts of HA detected in the bone marrow microenvironment using these fixation conditions (Figure 2D) The results indicate that tumor-associated HA is important for stimulating tumor growth in multiple tissue environments, and that HA is particularly critical for tumor cell survival/growth within the bone marrow microenvironment.

Hyaluronan could impact tumor growth *in vivo* by multiple effects on both the tumor and the tumor-associated stroma. We previously hypothesized that tumor-associated hyaluronan acts directly on tumor cells in an autocrine fashion to stimulate tumor growth [24]. To test this hypothesis, we adapted a methylcellulose assay to evaluate anchorage-independent growth of PC3M-LN4 prostate carcinoma cells *in vitro*. PC3M-LN4 cells express hyaluronan synthases, HAS2 and HAS3, and they also contain hyaluronidases that can cleave HA into smaller fragments. The parental cells growing in this assay form large multicellular colonies, similar to what is observed in agarose [32]. This assay offers advantages over agarose since the gels can be easily solubilized, allowing for recovery and quantification/biochemical characterization of cells at the end of the experiment. After 7 days of incubation, the cells were recovered from the gels and counted. The results show that PC3M-LN4 cells and mock-transfectants plated at low density ($3x10^4$/culture) within these matrices exhibit anchorage-independent growth over the 7 days of the assay (Figure 3). Inhibiting HA synthesis using HAS antisense vectors causes a significant inhibition of growth that can be reversed by the addition of highly purified hyaluronan (LifeCore, Chaska, MN). Reversal of growth inhibition was most notable in the presence of higher molecular weight HA (220 and 800 kD). The results are similar to what we observed *in vivo* following subcutaneous injection of tumors [25], leading to the conclusion that pericellular HA matrices synthesized by the tumor have a direct effect on stimulating metastatic prostate tumor growth *in vitro* and *in vivo*. These studies suggest that HA receptors on the tumor cells function to mediate HA stimulated growth, survival and invasion.

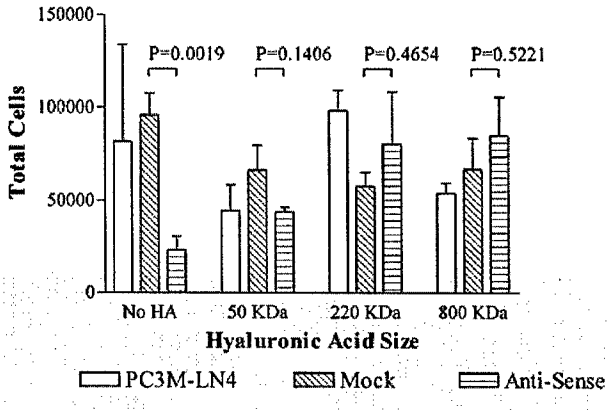

Figure 3. Growth restoration by exogenous HA in CaP HAS2/3 anti-sense cell line. Briefly, CaP cell lines were suspended in methylcelluose in the presence of 0.66 mg/mL HA of various sizes. After 7 days the cells were harvested and counted using trypan blue.

Hyaluronan as an Autocrine Factor for Prostate Tumor Growth: Tumor-Associated HA Receptors

The two most studied HA receptors are CD44 and Rhamm (Receptor for Hyaluronan Mediated Motility). Both of these receptors are implicated in tumor progression and metastasis, and have been shown to affect tumor adhesion, invasion, growth and survival [2, 12, 33]. CD44 and Rhamm are structurally unrelated and are encoded by unique genes located on distinct chromosomes (11p13 and 5q33, respectively). Despite their differing structures, 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 [12, 34]. 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 binding to other ECM ligands (fibronectin, collagen), binding/presenting specific growth factors and enhancing activation of their cognate signaling receptors [35, 36]. While some studies indicate that increased CD44 expression is a bad prognostic factor for certain cancers (e.g. renal cell cancer, papillary thyroid caricinoma and breast cancer), other cancers (e.g. head and neck, prostate cancer and ovarian cancer) appear to be characterized by decreased CD44 expression [34]. 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, 12, 34, 37, 38]. 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 examined.

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 [39-42]. 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 [41, 43]. This may help to explain apparently contradictory observations documenting increased CD44 levels in prostate tumor cells that have entered the circulation [44].

CD44 is also associated with the internalization of HA, which is important for hyaluronidase-mediated degradation of this high molecular weight glycosaminoglycan [45, 46]. Not surprisingly, loss of CD44 expression in inflammation models using CD44 -/- mice) results in enhanced accumulation of HA in the afflicted tissues with a corresponding increase in the severity of the inflammatory response [47]. 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 [12, 48-52]. The mechanisms by which Rhamm associates with the cell surface are not known; 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 [53]. 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 [12, 54, 55]. 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 [56]. Intracellular Rhamm 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 [12]. Intracellular hyaluronan and Rhamm have also been co-localized with elements of the mitotic spindle [2, 12, 15, 33, 48, 51], further supporting a function for Rhamm in cell cycle progression.

Rhamm expression is elevated in a number of tumors, including carcinoma of the breast, bladder, stomach, tumors of neural origin, and hematopoietic malignancies [57-60]. 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 [61]. While Rhamm seems important for the initiation of mesenchymal tumors, but not carcinomas [61], 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. This suggests 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 in the trabeculae and at the invasive edges of the tumor, implicating Rhamm overexpression in promoting invasion and metastasis [33]. While progression of transitional cell carcinoma of the bladder is associated with decreased levels of CD44 expression, Rhamm expression is positively correlated with progression of this tumor [58]. 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 tumor growth and survival [62-64]. Interestingly, 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 advanced disease (based on comparative Gleason scores) [65]. 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 of prostate tumors.

Conclusions

Dysregulated hyaluronan biosynthesis is associated with the progression of several malignant tumors, including prostate. Data from our laboratory and others implicate increased levels of hyaluronan in facilitating the growth, invasion and metastasis of prostate tumors. Furthermore, the results suggest that tumor associated receptors for hyaluronan are implicated in malignant behavior associated with increased levels of HA. Current efforts focus on identifying the importance of these hyaluronan receptors in the growth, survival and invasion of prostate tumor cells. One intriguing possibility is that multiple receptors for hyaluronan mediate the effects of this glycosaminoglycan on tumor growth and invasion at different stages of progression. Understanding the mechanisms by which hyaluronan facilitates tumor progression could yield

novel diagnostics and therapeutic agents that could be used in the management of patients with both early and advance prostate tumors.

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