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13. ABSTRACT (Maximum 200 Words)

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. Our studies 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. These cells also exhibit selective adhesion to bone marrow endothelial cell lines in vitro, suggesting that carcinoma associated HA may enhance entry of prostate tumor cells in to the bone marrow microenvironment by engaging specific receptors on the surface of these endothelial cells. Furthermore, elevated HA synthesis enhances tumor growth and vascularization in vivo following subcutaneous injection. 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 and 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.

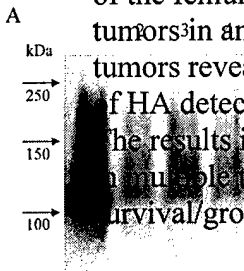


Figure 5. Western blot analysis for CD44s and RHAMM siRNA treated cells. PC3M-LN4 cells were treated with 20 μM of respective siRNA's for 48 h. The negative siRNA control (Lane 1) was ran as a control for both gels. Cells treated with siRNA's for CD44s were run in Lanes 2-5 (A) and siRNA's for RHAMM 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

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

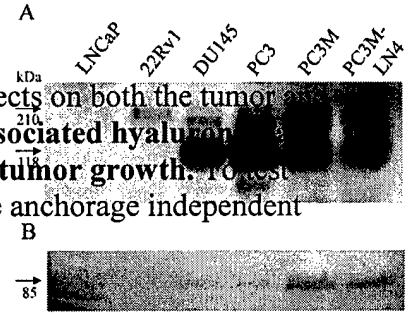
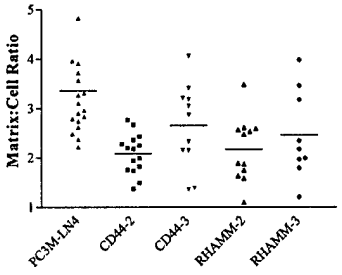


Figure 4. Western analysis of CD44 and RHAMM for prostate cell lines. Lysates from six prostate cancer cell lines were obtained run on a 12% SDS-PAGE then transferred to a nitrocellulose membrane. Membranes were blocked and probed with a 1:750 dilution of mouse hCD44H-(C25) (A) or 1:500 dilution of rabbit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

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*.

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 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.**



	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 pair-wise comparisions of interest.

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 initiated these studies as described above using a direct intrafemoral injection assay.

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

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

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

Month 36: Prepare final 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

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.

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.

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Appendix*

Nothing included for year 2

Final Reports

N/A (This is an annual report)