

AD _____

AWARD NUMBER DAMD17-96-1-6060

TITLE: Antagonistic Action of Hyaluronan Oligomers in Breast Cancer

PRINCIPAL INVESTIGATOR: Rebecca A. Moore Peterson

CONTRACTING ORGANIZATION: Tufts University
Boston, Massachusetts 02111

REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY. (Leave blank)		2. REPORT DATE September 1998		3. REPORT TYPE AND DATES COVERED Annual (1 Sep 97 - 31 Aug 98)	
4. TITLE AND SUBTITLE Antagonistic Action of Hyaluronan Oligomers in Breast Cancer				5. FUNDING NUMBERS DAMD17-96-1-6060	
6. AUTHOR(S) Rebecca A. Moore Peterson					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Tufts University Boston, Massachusetts 02111				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES					
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) The murine mammary carcinoma, TA3/St, grows aggressively in ascites and rapidly invades the peritoneal wall of syngeneic mice. The hyaluronan content of ascites increases markedly during TA3/St cell proliferation therein and hyaluronan is enriched at sites of attachment of these cells to the peritoneal wall. Thus we have investigated whether interference with interaction between hyaluronan and CD44 on the TA3/St cell surface, by overexpression of soluble CD44, would inhibit ascites tumor progression. Mice injected intraperitoneally with stable transfectants of TA3/St cells that overexpress soluble CD44 did not accumulate ascites fluid; the transfectants grew at a reduced rate within the peritoneum, then went into G1 arrest. However, transfectants overexpressing mutant soluble CD44 that does not bind hyaluronan exhibited similar ascites accumulation, growth rates and cell cycle profiles in vivo to wild type and vector-transfected TA3/St cells. The soluble CD44-transfected TA3/St cells also failed to attach and form tumors in the peritoneal wall. In contrast, wild type TA3/St cells, and transfectants carrying vector alone or mutant soluble CD44, formed such tumors rapidly and consistently. Thus, soluble CD44 acts as an antagonist of hyaluronan interactions that are essential for TA3/St tumor progression.					
14. SUBJECT TERMS Breast Cancer Murine Mammary Carcinoma Hyaluronan CD44 Invasion				15. NUMBER OF PAGES 21	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified		18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified		19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	
				20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

RMF In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985). Where copyrighted material is quoted, permission has been obtained to use such material.

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46. Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations. In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985). Where copyrighted material is quoted, permission has been obtained to use such material.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46. Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health. Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations. In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI Signature

Date

9/28/98

Antagonistic Action of Soluble CD44 and Hyaluronan Oligomers in Breast Cancer

Table of Contents

Front Cover.....	Page 1
SF 298.....	Page 2
Foreword.....	Page 3
Table of Contents.....	Page 4
Introduction.....	Page 5
Body.....	Page 8
Conclusions.....	Page 16
References.....	Page 18
Bibliography.....	Page 21

5) Introduction

Hyaluronan Influences Cell Behavior

Interactions between cells and their surrounding extracellular matrix are known to influence growth, migration, adhesion, and apoptosis of various cell types. Of particular importance is the role of matrix in stabilizing the differentiated state of cells. Subtle alterations in normal matrix properties can lead to aberrant cell division, escape from normal barriers to cell movement, and even cell death. One of the macromolecules commonly found in extracellular matrices is the polysaccharide, hyaluronan. Hyaluronan (HA) influences migration, growth and adhesion of cells through interaction with cell surface hyaluronan receptors (1-3). It is now known that in some tumor types the interactions of hyaluronan with these receptors is an essential component of the broad cascade of events necessary for tumor growth and metastasis (4-7), although investigation of the steps leading from hyaluronan-receptor interaction to intracellular alterations that induce aberrant cell behavior is still in its infancy. The influence of hyaluronan on cell behavior, and the mediation of these events by cell surface hyaluronan-binding proteins (HABPs), has long been the focus of investigations going on in our laboratory. These studies, as well as the results obtained in other laboratories, have demonstrated that the interaction of HA with many different cell types is likely to be fundamental to several aspects of cell behavior during embryonic development, tissue regeneration, and tumorigenesis (1-7).

Investigations into the effects of HA on cellular processes have shown: a) that HA is enriched in matrices in which cells migrate and proliferate in vivo; and, b) that HA influences cell behavior in vitro and in vivo (1-7). For example, HA stimulates movement of several cell types (10,13). Additionally, the inclusion of HA promotes cellular invasion of collagen gels, whereas the inhibition of the HA-HABP interaction, or the removal of endogenous HA by hyaluronidase treatment, blocks such invasion (14). HA has also been shown to mediate cell adhesion in several cell types (15,16), which may be vital to some aspects of embryonic development (17), tumor invasion and metastasis (18), and the immune response (19,20).

Cell Surface HABPs Mediate the Effects of Hyaluronan

Characterization of cell surface HABPs (HA receptors) that mediate HA-cell interactions is an area that has undergone dramatic expansion over the past several years. The best characterized HA receptors are CD44 and RHAMM. Several other HABPs have been identified but only RHAMM and CD44 have been convincingly shown to mediate cellular effects of HA. The CD44 gene produces an incredibly diverse group of proteins. At least 10 variant exons are alternatively spliced in various combinations into the extracellular region of the CD44 gene, and approximately 20 isoforms from various tissues and cell types have been isolated thus far (21,22). In addition, CD44 is posttranslationally modified by O- and N-glycosylation, glycosaminoglycan addition, and phosphorylation, all of which influence the reactivity of CD44 (23-25). The most predominant CD44 isoform is CD44H, and hyaluronan-CD44H interactions are thought to mediate endocytosis of hyaluronan (26,27) as well as various aspects of cell aggregation (16), cell adhesion (15,16), pericellular matrix assembly (8,9), and cell migration (10). CD44H acts as a major transmembrane HA receptor (28), and it is widely distributed in several tissue types. This standard form (CD44H), as well as all isoforms of CD44, include the HA-binding domain in their N-terminal regions despite their varying capacity to bind hyaluronan (21). Although the physiological functions of variant CD44 isoforms as HABPs remain unclear (3,20), they are mainly restricted to tumor cells, activated lymphocytes, and proliferating or morphogenetically active epithelia (22,29,30). Putative ligands for CD44 variants other than HA most likely exist, but their identities and functions are not well established.

Since cell surface HA-HABP interactions initiate many cellular effects of HA, the biochemical mechanisms by which these interactions are transduced into intracellular signals that bring about these effects are now being intensely studied by several groups. HA-CD44 interaction

causes an increase in intracellular Ca^{++} , clustering of CD44 in the membrane and accumulation of ankyrin beneath it (31,32,33). CD44 interacts with the tyrosine kinase, p56^{lck} , leading to increased phosphorylation of ZAP-70 and other intracellular proteins (34). CD44 also interacts with proteins associated with the cytoskeleton (31,35,36) and with lipids in the plasma membrane (37). Glycosylation of the extracellular domain (24) and integrity of the cytoplasmic tail (33,35,36,38) greatly influence HA binding. RHAMM also associates with cytoskeletal elements (39) and triggers a series of intracellular signaling events (40).

HA-HABP Interactions Modulate Cell Behavior During Tumorigenesis

The study of tumorigenesis is the most developed area of investigation implicating HA-HABP interactions in vivo. Several studies have shown: a) that invasive tumors produce elevated levels of HA in vivo compared to their benign counterparts (5,41); b) that direct and indirect interactions between tumor cells and stromal cells stimulate HA production by the latter (5,42,43); and c) that some metastatic tumor cells have much higher levels of cell surface HABP than their benign counterparts (44-46). It has recently become clear that CD44 and RHAMM are both involved in transformed and tumor cell migration, and various isoforms of CD44 and RHAMM are involved in tumor growth and metastasis. It has also become apparent that HA-CD44 and HA-RHAMM interactions lead to intracellular signaling events that modify cell behavior (31,47). Of particular interest is a recent study in which it was shown that HA initiates locomotion of ras-transformed fibroblasts via RHAMM-induced tyrosine kinase activity (47), and ultimately leads to metastatic behavior in vivo (6). A very rapid response to HA-RHAMM interaction is transient phosphorylation of p125^{FAK} in concert with turnover of focal adhesions (47). These events do not occur if HA-RHAMM interaction is suppressed, leading to stabilization of focal contacts and loss of motility (6). Transfection of CD44-negative lymphoma cells with cDNA for CD44H promotes tumor growth and metastasis (48). Administration of either antibodies that block HA binding to CD44H (49), soluble CD44-Ig fusion constructs that compete for HA-CD44H interaction (7), or HA oligomers that block HA-HABP interaction inhibits melanoma growth and/or metastasis (11). Furthermore, introduction of a single base mutation in CD44H that interferes with HA binding nullifies its efficacy in vivo (7). With respect to signaling, it appears likely that polymeric HA induces clustering of CD44 receptors (50) which may in turn be important for signal transduction, leading to modified cell behavior (31). Clustering of CD44 splice variants in rat carcinoma cells increases the binding affinity of soluble HA for CD44, thereby suggesting that HA binding is dependent of the ability of CD44 to aggregate in the plasma membrane (50). The loss of cell surface CD44 in colon carcinoma cells observed during CD95-induced apoptosis is also proposed as one of the first steps that may contribute to detachment of adherent cells during programmed cell death (51). Even though it can be seen that both CD44 and RHAMM play important roles in many aspects of tumor growth and progression, the focus of our project centers on HA-CD44 interactions during tumorigenesis.

HA oligomers and Soluble CD44 Block Tumor Growth

Past studies in our laboratory have shown that oligosaccharides of hyaluronan, as small as hexamers, competitively inhibit interaction of hyaluronan polymer with receptor. These oligomers also block migration of, and pericellular matrix assembly by, some embryonic cell types (8-10). Of particular interest is the finding that they inhibit formation of murine melanomas in vivo (11). The physiological action of hyaluronan polymer is likely to be mediated through clustering of its receptors, brought about by multivalent interaction of oligomeric sequences with multiple receptor units. Receptor clustering would then result in transmission of intracellular signals as happens with many other signal transducing, bi- or multi-valent, receptor-ligand interactions. We propose that hyaluronan oligomers act as antagonists that block hyaluronan polymer binding and resultant signal transduction. The latter would occur since the oligomers are too small to span several receptor units and thus would not induce the receptor clustering that may be necessary for signal

transduction. In addition, soluble CD44-Ig fusion proteins (7,10,13) and anti-CD44 antibodies (52,53) have also been shown to block progression of melanomas, lymphomas and ovarian carcinomas. Possible explanations of these findings could be that molecules that block the polymeric HA-HABP interaction, such as soluble CD44 and HA oligosaccharides, may interfere with this receptor clustering and resultant signal transduction. Our group has recently identified CD44 isoforms that are secreted due to lack of membrane and cytoplasmic domains (12), that may also have the capacity to act as antagonists of membrane CD44H (7,10,13). A newly described exon can be alternately spliced to create transcripts containing stop codons, thereby providing a molecular basis for *de novo* synthesis of a soluble CD44 variant. As with several other adhesion molecules, soluble CD44 has been detected in substantial amounts in serum, lymph, and synovial fluid from a variety of species (54). In addition, elevated levels of soluble CD44 have been detected in the sera of tumor patients (55-57). We hypothesize that antagonists to either membrane bound CD44H, such as soluble CD44, or to hyaluronan polymer, such as HA oligosaccharides, inhibit HA-CD44 interactions that are crucial to the cellular processes involved in breast cancer tumorigenesis (Figure 1). Consequently, we propose to investigate the inhibition of mammary

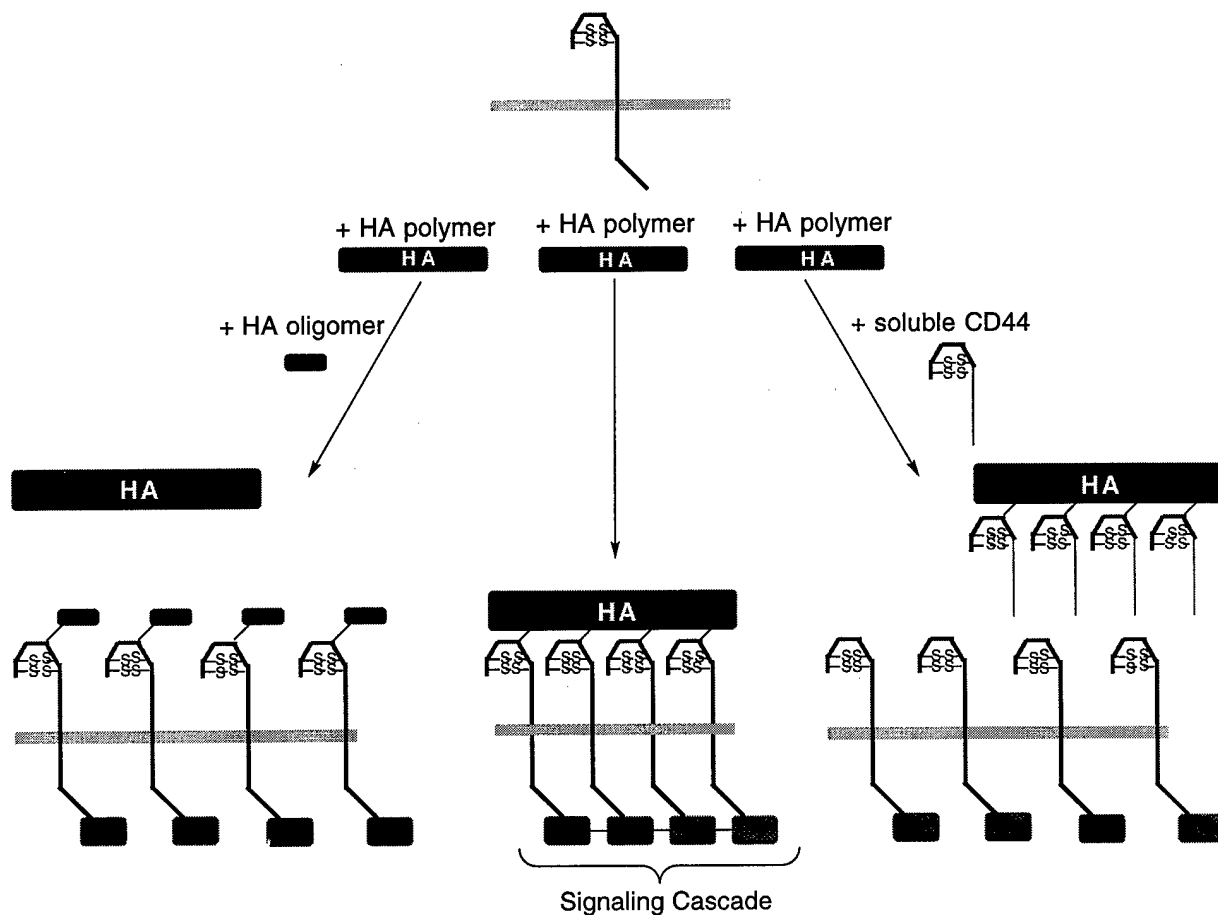


Figure 1: A model for the inhibition of hyaluronan-CD44 interactions by HA oligomers and soluble CD44.

tumor progression by soluble CD44 and HA oligomers in vivo. This report will focus on the effect of overexpression of soluble CD44 by transfection of tumor cells on murine mammary carcinoma growth and invasion in vivo within an intraperitoneal mouse model system.

Attachment Sites of CD44-Positive Tumor Cells Show Increased HA levels

Previous studies in our laboratory by Dr. Tet-Kin Yeo have investigated murine breast (TA3/St) and ovarian (MOT) ascites tumors as models to follow changes in HA levels during tumor growth, attachment and invasion in vivo. Ovarian and breast cancer cells frequently exfoliate into body cavities where they induce accumulation of ascites fluid to enable the tumor cells grow in suspension. A portion of these cells have been shown to attach and grow on mesenteric surfaces and consequently, especially in the case of breast cancer, invade these tissues. Subsequent to introduction of tumor cells into the peritoneal cavity, the amount of HA in the ascites rises from an undetectable level to peak values of 45-200 mg/l at 5 to 9 days post-injection. Using a specific probe to visualize HA, local HA accumulation was observed at initial sites of attachment of tumor cells to the surface mesentery (18). However, the tumor cell lines synthesize very low amounts of HA in culture. Therefore, it was concluded that the observed HA accumulation in vivo results from increased synthesis and secretion by mesothelial cells and/or fibroblasts, most likely in response to stimulation by direct interaction with the surface of tumor cells (42) or by their secreted products (43).

Virtually all of the TA3/St tumor cells that initially attach to mesentery are strongly positive for CD44. At later stages both CD44-positive and negative cells accumulate within the peritoneal wall. Following attachment, the TA3/St mammary tumor cells invade the mesentery and HA is deposited at the tumor-host interface. Thus, it was proposed that an HA-rich matrix promotes tumor cell attachment and invasion by interaction with tumor cell surface CD44 (18). As mentioned previously, this is supported by studies demonstrating inhibition of attachment of ovarian carcinoma cells to mesothelium in vitro and in vivo by antibody to CD44 (52,53) and inhibition of melanoma progression in vivo by soluble CD44-Ig fusion constructs (7). Therefore, we decided to further test the mediation of murine mammary (TA3/St) tumorigenesis by HA-CD44 interactions between the tumor cells and the surrounding HA-rich matrix found in this model system.

6) Body

Experimental Methods

Cells and cell culture. TA3/St tumor cells were maintained by weekly passages in the peritoneal cavities of syngeneic, 4 to 6-week-old female A/Jax mice or in culture in Dulbecco's modified Eagle's medium (DMEM: Gibco BRL, Life Technologies) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories Inc.). Cell lines transfected with soluble CD44 or vector alone were maintained in DMEM supplemented with 10% fetal bovine serum and 500µg/ml Geneticin (G418 Sulfate, Gibco BRL, Life Technologies).

Transfection of TA3/St Cells with Soluble CD44 Constructs. Soluble CD44 constructs were prepared and analyzed as described previously (58). For transfection, TA3/St cells were treated with either pCR3-Uni vector containing cDNAs encoding soluble CD44 isoforms in the presence of Lipofectamine. These isoforms included either variant exons v8-v10 or v6-v10, where v10 is a new insert containing a stop codon thus leading to truncation prior to the transmembrane domain (12,58); v6-v10 was used with or without the R43A mutation that leads to loss of hyaluronan binding capacity (58). G418 resistant colonies were selected and six clones were chosen for further study: two transfectants containing v6-v10 (v6-v10a and v6-v10b), one containing v8-v10, one containing the v6-v10 mutant (v6-v10 R43A), and two mock transfectants containing vector only (C1 and C8).

Tumorigenicity Assay. TA3/St cells in log phase growth were trypsinized, washed with DMEM containing 10% FBS, and resuspended in injection media (HBSS, Gibco BRL, Life Technologies) for counting. Suspensions of TA3/St cell lines were injected, using a 25 gauge needle, into the peritoneal cavities of 4 to 6-week old female A/Jax mice at 1×10^6 cells/200 μ l of injection media each and allowed to grow in vivo for a period of 7 to 19 days. For each cell line and time point, 6 mice were given injections. Mice were observed daily for signs of ascites tumor development and monitored twice daily after the following tumor symptoms appeared: abdominal bloating, decreased movement, loss of grooming behavior, and hunched posture. If mice were not expected to survive overnight, they were sacrificed prior to conclusion of the experimental protocol. Mice that did not exhibit the above symptoms were sacrificed according to experimental parameters. The peritoneal walls from each of the mice were removed, cut into strips (approximately 4mm x 8mm), and fixed in 4% paraformaldehyde (Tousimis, Rockville, MD) in phosphate-buffered saline (PBS) for histological analysis.

Histology. Fixed strips of peritoneal wall were washed in PBS, dehydrated through a series of ethanol (30%, 70%, 95%, 100%) and xylene washes and embedded lengthwise in paraffin wax blocks to ensure that the cut sections would be cross-sections of the mesothelium and the muscles of the peritoneal wall. Sections (5 μ m) were cut, mounted on Poly-L-Lysine (Sigma) coated slides and stained with hemotoxylin and eosin (Richard-Allen Medical, Richland, MI) after deparaffinization in xylene and rehydration through 100%, 95%, 70%, 35% ethanol, PBS and water.

In Vivo Cell Proliferation Assay. Transfected TA3/St cells in log phase growth were trypsinized, washed with DMEM containing 10% FBS, and resuspended in injection media for counting. Suspensions of the transfected TA3/St cells were seeded into the peritoneal cavities of female A/Jax mice at 2×10^6 cells/200 μ l of injection media each and allowed to grow in vivo for a period of 2 to 15 days. At each of five different time points (2, 5, 7, 10 and 15 days), groups of 6 mice were sacrificed and cells were harvested from the peritoneal cavities with two 6 ml intraperitoneal lavages of calcium and magnesium-free PBS (PBS⁻, Gibco BRL, Life Technologies). Harvested cells were then washed with PBS⁻ to remove any red blood cells that were withdrawn along with tumor cells from the peritoneal cavity, and the washed cells were counted in a Coulter Counter (Hialeah, FL) by diluting aliquots of cells resuspended in PBS⁻ to concentrations between 200 to 20,000 cells/ml.

Cell Cycle Analyses. Transfected and wild type TA3/St cells were grown intraperitoneally for seven days, harvested and washed as described in the previous section. They were then resuspended in 70% EtOH and kept at -20°C until all samples had been collected for cell cycle analysis. After removal from the freezer, the cells were washed twice with PBS⁻, resuspended in PBS⁻ containing 0.1 mM EDTA pH 7.4, 50 mg/ml propidium iodide, 50 mg/ml RNase A (Boehringer Mannheim) and 1% Triton X-100, and incubated overnight at 4°C. Cell samples were then analyzed by fluorescence activated cell sorting in a FACScan[®] (Becton Dickinson, Mountain View, CA). Transfected and wild type cells analyzed following culture in vitro were maintained in DMEM supplemented with G418 and 10% FBS or with 10% FBS alone, respectively, then harvested during log phase of growth and analyzed in the same way as described above.

Results and Discussion

Transfection of TA3.St Cells with Soluble CD44 cDNA

As mentioned previously, our group has identified CD44 isoforms that are secreted due to lack of membrane and cytoplasmic domains (12). These soluble isoforms arise because of stop codons found in three newly discovered inserts, or exonic sequences, that can be alternatively

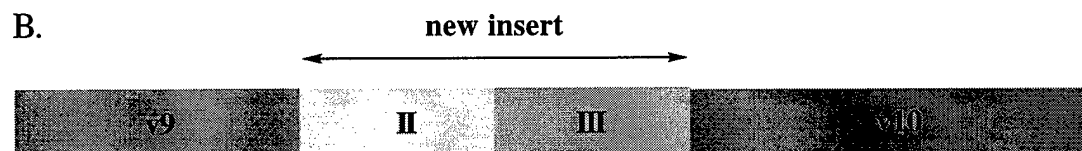
spliced to create these soluble forms of the CD44 protein (Figure 2a). Since soluble CD44 would be expected to antagonize the interaction between hyaluronan and membrane bound CD44H, we investigated whether stable transfection of malignant tumor cells with cDNA encoding one of these soluble CD44 isoforms (Figure 2b) would prevent their ability to form tumors *in vivo*. We used the above-described murine mammary carcinoma line (TA3/St) which grows in ascites form and metastasizes through the peritoneal wall of syngeneic mice. As stated above, studies in our lab have previously shown that these cells express CD44 and attach to regions of the peritoneal wall enriched in hyaluronan prior to invasion through the wall (18).

Stable transfectants expressing soluble CD44 isoforms (v6-v10a (#63A), v6-v10b (#63B), v8-v10), mutant soluble CD44 isoform (v6-v10 R43A), or vector alone (C1 and C8) were selected and analyzed by RT-PCR and Western blotting techniques to confirm that each clone expresses the appropriate CD44 transcript, as well as to show that the protein was indeed being produced and secreted (58). The transfectants were then grown in media enriched with G418 sulfate that would ensure the selection of subsequent generations in culture that carry the *neo* vector cDNA. The soluble CD44 transfectants were shown to secrete CD44 whereas the transfectants carrying vector alone did not (58). All transfectants and wild type cells produced membrane-associated standard CD44H (58).

A.

GTAAGGATCATAAAGTCCAAGTGGCTTTTAAGCAGAAATCAAGACGTTAT
 GGGTGTGTCTGGTGGTGGTTGCTGATTTTCTGCTTTATAGATCTTTTAGAAG
 CCATTGTACACTAG GTACAAACTTTTCTGCTGAATTTTAAAG

B.



Amino acid sequence of new insert: VCLVVVADFSAL***

Figure 2: **Sequence and arrangement of new insert between v9 and v10.**

A, Various combinations of three novel inserts give rise to soluble isoforms of CD44. These inserts are designated: I, II, and III. Stop codons that result from these inserts are marked with triple asterisks (***); the particular stop codon introduced depends on the combination of inserts used [Yu and Toole, (1996) *J. Biol. Chem.* 271: 20603-20607]. B, The cDNA used for producing stable transfectants contained the variant exons, V6, V7, V8, V9 and inserts II and III. Two separate clones obtained from the transfection were used: 63A and 63B.

Overexpression of Soluble CD44 Inhibits Tumorigenicity of TA3/St Cells *in Vivo*

Transfected TA3/St cells overexpressing soluble CD44 (v6-v10a, v6-v10b) were injected intraperitoneally into syngeneic female A/Jax mice and allowed to grow within the peritoneal cavity of these mice for 7 to >14 days post injection. In parallel control experiments, wild type TA3/St cells and TA3/St cells transfected with vector alone (Controls 1 and 8) were also injected in the same concentrations. We evaluated several animals in each of the above conditions by determining the degree of ascites growth and TA3/St cell attachment and invasion into the peritoneal wall, and the results are summarized in Table 1. We found that TA3/St cells overexpressing soluble CD44

usually failed to form tumors in the peritoneal wall or mesentery whereas wild type TA3/St cells or transfectants carrying vector alone or mutant soluble CD44 (v6-v10 R43A) formed tumors rapidly and consistently. At 7 days post-injection, tumor cells in animals injected with control (vector alone) transfectants or wild-type TA3/St cells can be seen to line up along the mesothelium as they attach to the inside of the peritoneal wall (Figure 3a). Yet, in animals injected with transfectants overexpressing soluble CD44 (Figure 3b), the mesothelium remains relatively smooth and unchanged. Likewise, after a period exceeding 14-19 days post-injection, mice carrying TA3/St transfectants overexpressing soluble CD44 are also seen to have peritoneal walls that appear generally unaffected (Figure 4b) when compared to the peritoneal walls of mice injected with wild-type, mock-transfected or mutant soluble CD44-transfected (v6-v10 R43A) cells exhibited widespread tumorigenesis and invasion of the mesothelium and muscle layers of the peritoneal walls (Figure 4a, c, d). Yet, it should be noted that transfectants were found to attach to a limited extent to the peritoneal wall of three out of 12 mice sacrificed at >14 days post-injection. However, the transfectants attached at a few discrete foci only, whereas the controls formed continuous masses of tumor cells along and within the mesothelium and peritoneal wall. In these three cases small tumors were also observed, but they were isolated and surrounded by intact mesothelium very unlike the tumors seen in the tissues from control animals that exhibited widespread and uniform invasion throughout the peritoneal wall. In addition, unlike mice injected with wild type, mock-transfected or mutant soluble CD44-transfected (v6-v10 R43A) cells (Figure 5a), the mice injected with soluble CD44 transfectants do not accumulate ascites (Figure 5b), indicating that tumor cell growth therein is also markedly reduced.

Table 1:

Reversal of Tumorigenicity in TA3/St Mammary Carcinoma Transfectants					
Cell Type	Time Elapsed Before Analysis	# of Mice	Attachment at 7 days	Invasion >14 days*	Ascites Growth
Controls:					
TA3/St w/t	7 days	6	6	na	na
	>14 days*	6	na	6	6
TA3/St vector alone	7 days	6	6	na	na
	>14 days*	6	na	6	6
Transfectants:					
TA3/St v6-v10a	7 days	6	0	na	na
	>14 days*	6	na	1 [#]	0
TA3/St v6-v10b	7 days	6	0	na	na
	>14 days*	6	na	2 [#]	0

* Animals were examined between 14 and 19 days after injection.

[#] Limited and inconsistent attachment; small tumors or foci regions of tumor cell accumulation.

na: Not applicable for the mice sacrificed at this time point.

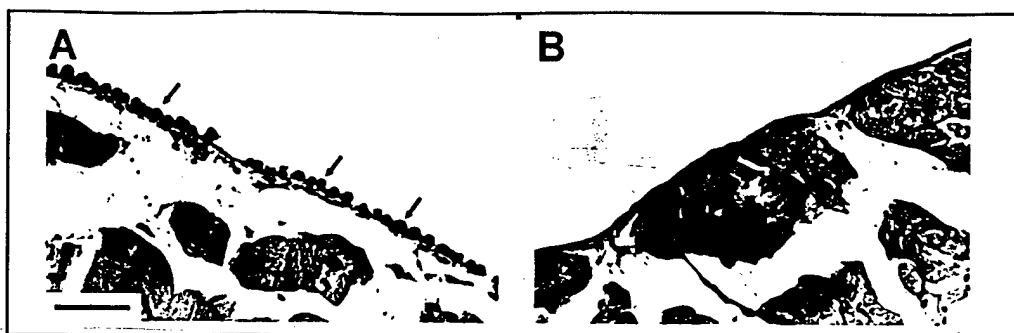


Figure 3. Inhibition of cell attachment to the peritoneal wall in mice injected with soluble CD44 transfectants. TA3/St transfectants were injected into the peritoneal cavity of syngeneic A/Jax mice, than the animals were sacrificed after 7 days, and their peritoneal walls were fixed and stained. **A.** Control TA3/St cells transfected with vector alone; **B.** TA3/St cells transfected with soluble CD44. The control cells attach to the peritoneal wall (arrows) whereas the soluble CD44 transfectants did not. Similar attachment to that shown in **A** for mock-transfectants was also obtained with wild-type and mutant soluble CD44 (R43A)-transfected cells (not shown). Scale bar = 50 μ m.

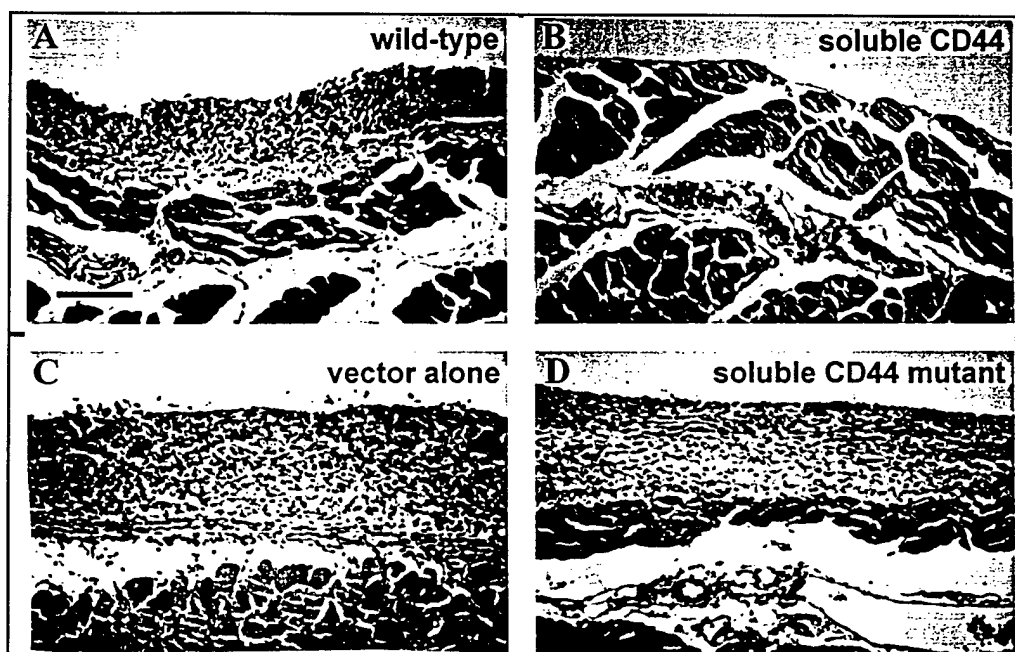


Figure 4. Reversal of tumorigenicity in soluble CD44 transfectants. TA3/St transfectants overexpressing soluble CD44, mock transfectants (vector alone), transfectants carrying soluble CD44 with a point mutation (R43A) in the hyaluronan-binding domain (mutant soluble CD44) or wild-type TA3/St murine mammary carcinoma cells were injected into the peritoneal cavity of syngeneic mice. At 14-17 days post-injection, the mice were sacrificed, and their peritoneal walls were fixed and stained. Mice injected with wild-type (**A**), mock-transfected (**C**) or mutant soluble CD44-transfected cells (**D**) exhibited widespread tumorigenesis and invasion of the mesothelium and muscle layers of their peritoneal walls. Mice injected with soluble CD44-transfected cells (**B**) were shown to have normal peritoneal walls. Scale bar = 100 μ m

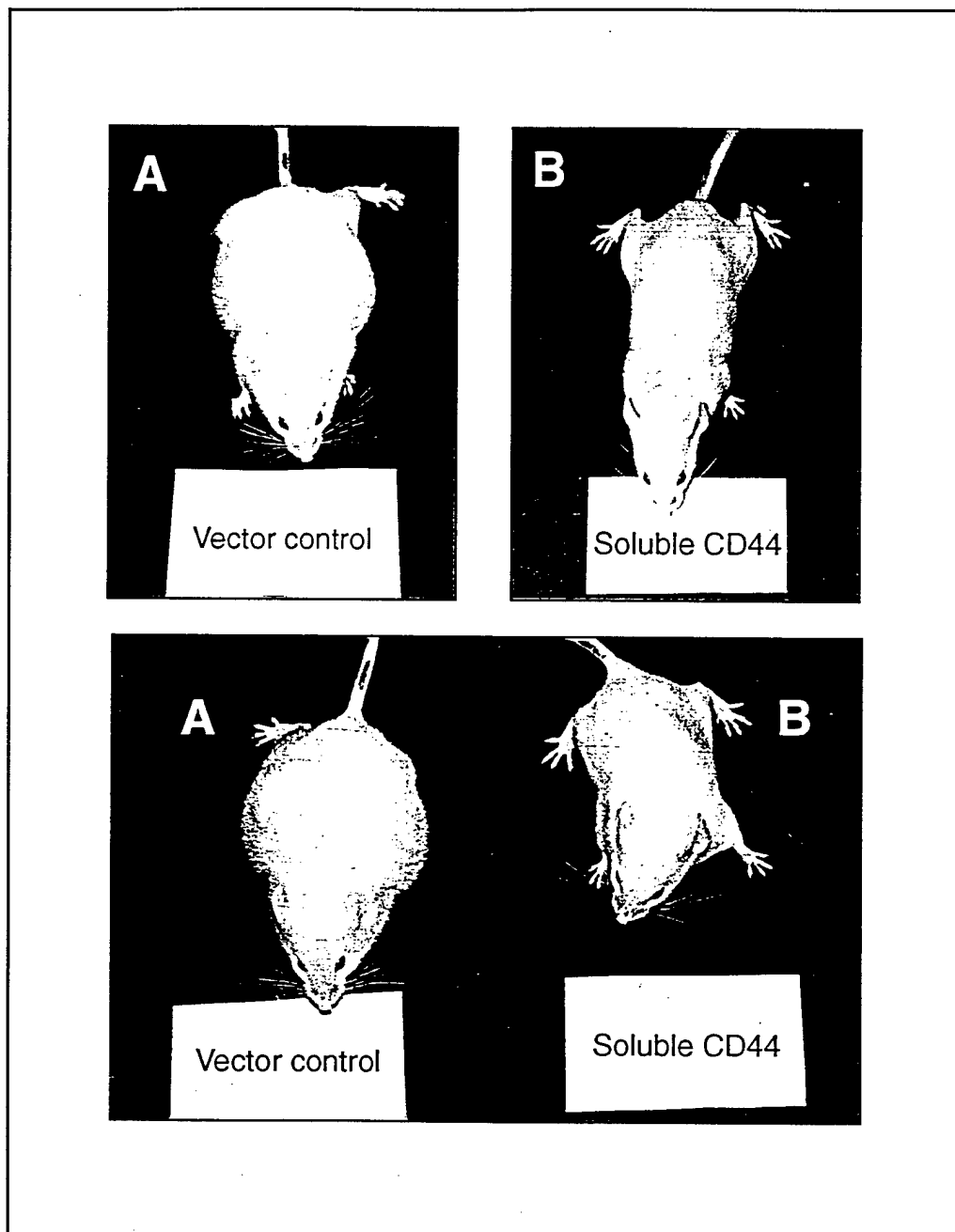


Figure 5. Mice injected with transfectants overexpressing soluble CD44 do not accumulate ascites fluid. A, control mouse injected with mock-transfected TA3/St cells and photographed after 18 days. B, mouse injected with transfectant, v6-v10a, and photographed after 18 days. The abdominal swelling in the control mouse is due to accumulation of ascites fluid caused by growth of the TA3/St tumor cells in the peritoneum. This swelling is not seen in mice injected with v6-v10a-transfected cells (above) or with v6-v10b or v8-v10-transfected cells (not shown). Yet, ascites accumulation is observed in mice injected with wild-type, mock-transfected and mutant soluble CD44-transfected cells as well (not shown).

Growth of Soluble CD44 Transfectants in Vivo

Since transfection with soluble CD44 cDNA eliminated ascites accumulation and invasion of the peritoneal wall, we followed growth of the injected cells in an attempt to determine their fate in vivo. To facilitate high cell recovery from the peritoneal cavity, a larger number of cells, 2×10^6 per animal, were injected into the abdomen of the host mice than in the experiments above. The cells were recovered by intraperitoneal saline lavage at 2, 5, 7, 10 and 15 days post-injection and counted (Figure 6). The mock-transfected and soluble CD44 R43A-transfected cells grew rapidly for the first 10 days after injection; however, most of these animals then became terminally ill and were sacrificed prior to day 15. Consequently only one to two animals remained for analysis at the 15-day point in these cases (in all other situations at least 6 animals were analyzed at each of the 5 time points). For the first 5-7 days, the soluble CD44 transfectants grew significantly, but at a diminished rate compared to the controls. Subsequently, between 10 and 15 days post-injection, their numbers became reduced to baseline (Figure 6). This rise and fall in number of cells is particularly evident for the transfectant overexpressing the v8-v10 soluble CD44 isoform, where the ascites cell number rose to approximately 14×10^6 at day 7 after injection but fell back to baseline by 15 days (Figure 6). None of the animals carrying the soluble CD44 transfectants developed ascites tumors and most survived indefinitely without any signs of tumor formation. A few of these animals slowly developed solid tumors outside the peritoneum near the site of tumor cell injection, presumably arising from cells that leaked from the peritoneum during injection or healing. From past experience, it is likely that these tumors arose from cells that had ceased to express soluble CD44 (58).

Cell Cycle Analysis of Soluble CD44 Transfectants in Vivo and in Vitro

Wild-type and transfected TA3/St cells were harvested at seven days post-injection from the peritoneal cavity of mice injected with 2×10^6 cells. These cells were analyzed by fluorescence activated cell sorting to establish a cell cycle profile for each cell line in vivo. Cells transfected with soluble CD44 isoforms exhibited G1 arrest, whereas wild-type, mock-transfected, and soluble CD44 R43A-transfected cells demonstrated a cell cycle profile typical of an asynchronous cycling cell population (Figure 7; Table 2). The proportion of cells in G0/G1 for each population was calculated to be approximately 30-40% for the various control populations compared to 75-85% for the soluble CD44 transfectants (Table 2). In culture, both the soluble CD44 transfectants and the control cell types exhibited similar cell cycle profiles, ranging from approximately 30-50% cells in G0/G1 (Table 2). In a parallel study, we have compared the ability of the soluble CD44-transfected and vector-transfected TA3/St cells studied herein to form metastases in the lung after intravenous injection (58). In that study, overexpression of soluble CD44 was shown to induce apoptosis subsequent to entry of the cells into lung tissue, and consequently growth of metastatic nodules was dramatically inhibited. In the current study it is also possible that the soluble CD44 transfectants became apoptotic. Consequently, we analyzed the cells obtained from the ascites at 5-7 days post-inoculation for morphological or biochemical signs of apoptosis, but we did not observe such characteristics. Thus, we cannot be certain whether loss of the soluble CD44 transfectants was due to apoptosis or necrosis since they were cleared efficiently and were unavailable for further analysis. TA3/St cells transfected with mutated soluble CD44 (R43A) behaved like vector-transfected controls, indicating that an hyaluronan-mediated interaction is involved in these effects of soluble CD44 on growth. Currently, we are investigating this cell cycle arrest phenomenon and attempting to identify cell cycle regulators that may be affected by the introduction of soluble CD44 into these cells, or the loss of hyaluronan-mediated events in this system.

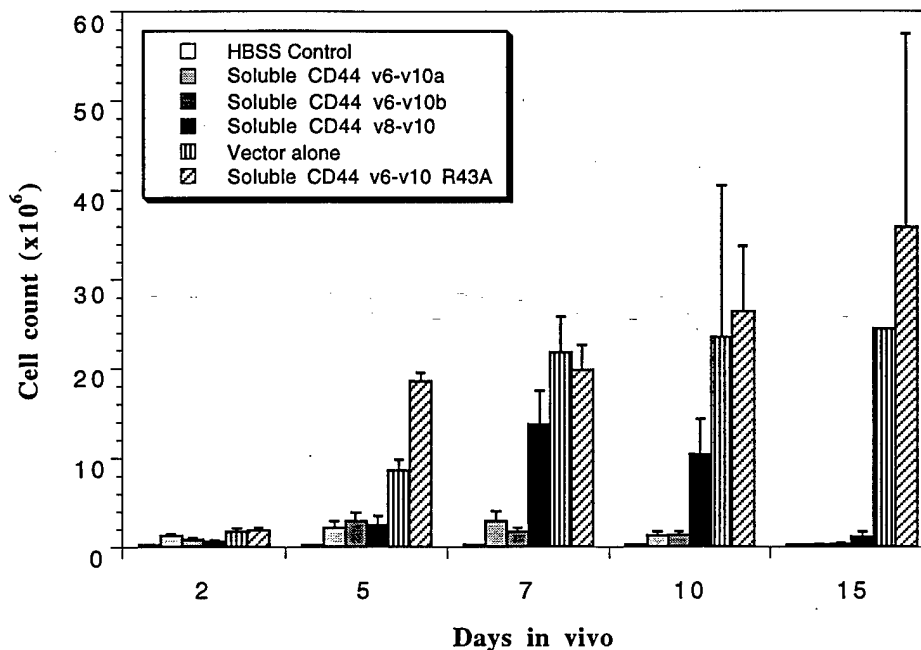


Figure 6. Cell growth of soluble CD44 transfected cells in ascites in vivo. Cell numbers were counted for soluble CD44-transfected, mock-transfected and soluble CD44 R43A-transfected TA3/St cells over 15 days in vivo. The soluble CD44 transfecteds (v6-v10a, v6-v10b, v8-v10) grow at a diminished rate and drop back to baseline (HBSS control) between 10 to 15 days post-injection, whereas the mock-transfected (vector alone) and the mutant soluble CD44 R43A-transfected cells continue to grow. Growth rates of cells within the peritoneal cavity of six mice per condition and time point are represented, except in the case of the mock-transfected and soluble CD44 R43A-transfected cells at 15 days post-injection, where many of the animals became ill and had to be sacrificed prior to day 15.

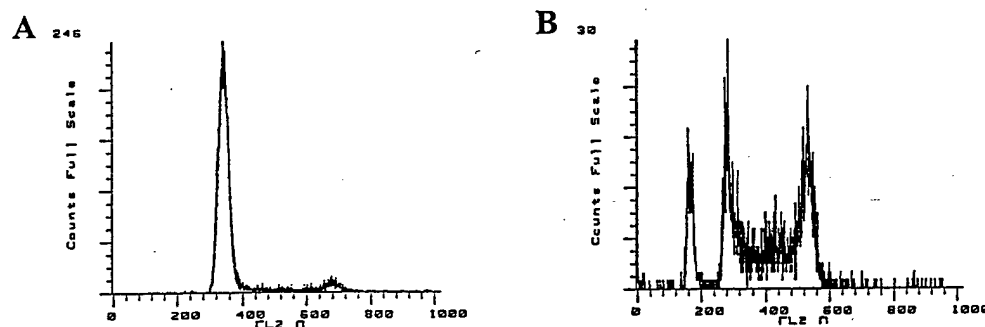


Figure 7. Cell cycle analyses of soluble CD44 transfected cells grown in ascites in vivo. **A.** Soluble CD44-transfected cells; **B.** Vector-transfected control. Quantitative data are given in Table 2. A similar cell cycle pattern to that of the vector-transfected cells (**B**) was obtained with wild-type and mutant soluble CD44 R43A-transfected cells (see Table 2).

Table 2:

Soluble CD44 Transfectants Exhibit G1 Arrest In Vivo			
Cell Type	% Cells in G1		In Vivo
	In Vitro		
<i>Controls:</i>			
TA3/St w/t	44.1 ± 0.5		30.0 ± 2.1
TA3/St vector alone	28.3 ± 1.5		38.2 ± 5.4
Soluble CD44 v6-v10 R43A	33.3 ± 0.9		39.1 ± 4.8
<i>Transfectants:</i>			
Soluble CD44 v6-v10a	44.7 ± 1.6		86.2 ± 1.0
Soluble CD44 v6-v10b	37.0 ± 2.1		76.4 ± 4.1
Soluble CD44 v8-v10	53.0 ± 1.2		74.3 ± 2.8

7) Conclusions

HA-HABP interactions have been implicated in several different investigations of tumorigenesis in vivo. Various studies have demonstrated that HA levels are elevated in vivo by highly invasive tumors (5,18,41), and that some metastatic tumors have more HABPs than their benign counterparts (44-46). Furthermore, antagonists to the HA-HABP interaction, especially in the case of HA-CD44 interaction, have been shown to abate tumor progression both in vitro and in vivo. For example, soluble CD44-Ig fusion proteins (7), and HA oligosaccharides (11), have been shown to block the progression of melanomas. In addition, the administration of antibodies against CD44 significantly reduced the number of ovarian cancer implants within a nude mouse xenograft intraperitoneal model (53) that is similar to the system employed in our studies. Since both ovarian and breast cancer cells often exfoliate into body cavities where they induce the accumulation of ascites fluid necessary for their survival and growth, an intraperitoneal model is an excellent environment for studying the tumorigenicity and metastatic potential of breast carcinomas. Therefore, by evaluating the behavior of these cells in this animal system, we have been able to gain insight into which extracellular matrix components may be contributing to the highly metastatic and lethal nature of these cells.

In this study we have demonstrated that the transfection of a TA3/St breast carcinoma cell line with cDNA for soluble CD44 inhibits the tumorigenicity of these cells in a syngeneic murine intraperitoneal model system. The work presented here demonstrates that stable transfection of malignant TA3/St cells alters their growth and adhesion characteristics in vivo such that they are unable to form ascites tumors or to invade the tissues of the host animal following intraperitoneal injection. Since transfection with mutated cDNA, encoding soluble CD44 that does not bind hyaluronan, fails to inhibit the tumorigenicity of TA3/St cells, soluble CD44 most likely acts by competitively disrupting an interaction involving hyaluronan.

A particularly striking finding of this study was the failure of TA3/St transfectants overexpressing soluble CD44 to form ascites tumors. For each of the three soluble CD44 transfectants tested, growth took place for several days in the peritoneum subsequent to inoculation. However, the rate of growth of the soluble CD44 transfectants was slower than for vector-transfected controls and the former cells went into G1 arrest, whereas the control cells continued to increase in number to a point that became fatal for the host animals. Growth of the soluble CD44-transfected cells not only ceased but the numbers of cells in the ascites decreased to unsubstantial levels. Depending on the particular transfectant, 3-14 million cells per mouse were lost from the peritoneum between 5 and 15 days post-inoculation, implying that the soluble CD44 transfectants not only went into G1 arrest but subsequently died and were cleared from the peritoneal cavity. As discussed above, it is possible that these cells followed an apoptotic pathway, as we observed in a parallel study (58), however we were unable to observe signs of apoptosis in our system since it is likely that apoptotic cells were rapidly eliminated from the peritoneal cavity upon cell death.

Interestingly, the cell number reached in the peritoneum for the soluble CD44 transfectants, 5-7 days after inoculation, was sufficient for widespread attachment to the peritoneal wall to occur in the case of the controls. However, no attachment of soluble CD44 transfectants was detected. This observation suggests that perturbed hyaluronan-CD44 interactions lead both to altered growth characteristics within the ascites and to inhibition of peritoneal wall implantation. Previous studies implicate interactions between tumor cell surface CD44 and mesothelial cell-derived hyaluronan in tumor cell attachment to the peritoneal wall (18, 43, 52, 53). Thus, it could be anticipated that overexpression of soluble CD44 would antagonize attachment of the TA3/St cells to the wall in our study. Therefore, together with our previous work, the results of this study imply strongly that soluble CD44 acts in this system as an antagonist of HA-mediated interactions that are essential for TA3/St tumor progression.

In summary, our results provide evidence that the HA-mediated interactions are involved in the growth, attachment and invasion of breast cancer cells *in vivo*, and that the inhibition of HA interactions by soluble CD44, or other potential antagonists, may be a useful strategy for reducing the spread of this disease. Since hyaluronan-receptor interactions are essential in many cases of tumor progression, soluble CD44 may be especially useful for treatment of breast cancer cases, where self examination and other diagnoses often reveal mammary tumors prior to metastasis. In some of these cases HA-HABP antagonists, like soluble CD44, could be administered locally subsequent to surgery to prevent further tumor progression and invasion into the host tissues. Therefore, the continuing goal of our research will be to focus on identifying other antagonists, such as hyaluronan oligosaccharides, that effectively interrupt the HA-CD44 interaction and significantly limit tumor progression and metastasis of breast carcinomas. Further studies will also include the identification of a mechanism of action for the inhibition caused by the introduction of soluble CD44 into this model system; thereby elucidating other targets and approaches that could form the basis of new therapies.

8) References

1. B. P. Toole, in *Cell Biology of Extracellular Matrix: Proteoglycans and hyaluronan in morphogenesis and differentiation*, E. D. Hay, Ed. (Plenum Press, New York, ed 2, 1991), pp 305-341.
2. E. A. Turley, *Cancer Metast. Rev.* **11**, 21 (1992).
3. L. Sherman *et al.*, *Current Opinion Cell Biol.* **6**, 726 (1994).
4. J. Ziegler, *J. Nat. Canc. Inst.* **88**, 397 (1996).
5. W. Knudson *et al.*, *Ciba Found.* **143**, 150 (1989).
6. C. L. Hall *et al.*, *Cell* **82**, 19 (1995).
7. A. Bartolazzi *et al.*, *J. Exp. Med.* **180**, 53 (1994).
8. W. Knudson, E. Bartnik, C. B. Knudson, *Proc. Nat. Acad. Sci. USA* **90**, 4003 (1993).
9. C. B. Knudson, *J. Cell Biol.* **120**, 825 (1993).
10. L. Thomas *et al.*, *J. Cell Biol.* **118**, 971 (1992).
11. C. Zeng *et al.*, (1997), submitted.
12. Q. Yu and B.P. Toole, *J. Biol. Chem.* **271**, 20603 (1996).
13. L. Thomas *et al.*, *J. Invest. Dermatol.* **100**, 115 (1993).
14. S. Koocheckpour, G. J. Pilkington, A. Merzak, *Int. J. Cancer* **63**, 450 (1995).
15. C. B. Underhill and B. P. Toole, *Exp. Cell Res.* **131**, 419 (1981).
16. S. J. Green, G. Tarone, C. B. Underhill, *Exp. Cell Res.* **178**, 224 (1988).
17. C. B. Knudson and B.P. Toole, *Develop. Biol.* **124**, 82 (1987).
18. T.-K. Yeo *et al.*, *Amer. J. Pathol.* **148**, 1733 (1996).
19. K. Miyake *et al.*, *J. Exp. Med.* **172**, 69 (1990).
20. I. Stamenkovic *et al.*, *EMBO J.* **10**, 343 (1991).
21. G. R. Screato *et al.*, *Proc. Nat. Acad. Sci. USA* **89**, 12160 (1992).
22. C. R. Mackay *et al.*, *J. Cell Biol.* **124**, 71 (1994).
23. L. Y. Bourguignon *et al.*, *Mol. Cell. Biol.* **12**, 4464 (1992).
24. A. Bartolazzi *et al.*, *J. Cell Biol.* **132**, 1199 (1996).
25. K. L. Bennett *et al.*, *J. Cell Biol.* **128**, 687 (1995).

26. C. B. Underhill *et al.*, *Develop. Biol.* **155**, 324 (1993).
27. Q. Hua, C. B. Knudson, W. Knudson, *J. Cell Sci.* **106**, 365 (1993).
28. A. Aruffo *et al.*, *Cell* **61**, 1303 (1990).
29. U. Gunthert *et al.*, *Cell* **65**, 13 (1991).
30. W. Rudy *et al.*, *Cancer Res.* **53**, 1262 (1993).
31. L. Y. Bourguignon *et al.*, *J. Immunol.* **151**, 6634 (1993).
32. E. Galluzo *et al.*, *Eur. J. Immunol.* **25**, 2932 (1995).
33. C. F. Welsh, D. Zhu, L. Y. Bourguignon, *J. of Cell Physio.* **164**, 605 (1995).
34. E. Taher *et al.*, *J. Biol. Chem.* **271**, 2863 (1996).
35. A. Perschl *et al.*, *Eur. J. Immunol.* **25**, 495 (1995).
36. V. B. Lokeshwar, N. Fregien, L. Y. Bourguignon, *J. Cell Biol.* **126**, 1099 (1994).
37. S. J. Neame *et al.*, *J. Cell Sci.* **108**, 3127 (1995).
38. J. Lesley *et al.*, *J. Exp. Med.* **175**, 257 (1992).
39. E. A. Turley, P. Brassel, D. Moore, *Exp. Cell Res.* **187**, 243 (1990).
40. E. A. Turley, *J. Biol. Chem.* **264**, 8951 (1989).
41. B. P. Toole, C. Biswas, J. Gross, *Proc. Nat. Acad. Sci. USA* **76**, 6299 (1979).
42. W. Knudson, C. Biswas, B. P. Toole, *Proc. Nat. Acad. Sci. USA* **81**, 6767 (1984).
43. T. Asplund *et al.*, *Cancer Res.* **53**, 388 (1993).
44. R. E. Nemec, B. P. Toole, W. Knudson, *Bioch. Biophys. Res. Comm.* **149**, 249 (1987).
45. T. Asplund and P. Heldin, *Cancer Res.* **54**, 4516 (1994).
46. N. Iida and L. Y. Bourguignon, *J. Cell. Physio.* **162**, 127 (1995).
47. C. L. Hall *et al.*, *J. Cell Biol.* **126**, 575 (1994).
48. M. S. Sy, Y.-J. Guo, I. Stamenkovic, *J. Exp. Med.* **174**, 859 (1991).
49. Y. Guo *et al.*, *Cancer Res.* **54**, 1561 (1994).
50. J. Sleeman *et al.*, *J. Cell. Biol.* **135**, 1139 (1996).
51. A. R. Gunthert *et al.*, *J. Cell. Biol.* **134**, 1089 (1996).
52. S. A. Cannistra *et al.*, *Cancer Res.*, **53**, 3830 (1993).

53. T. Strobel, L. Swanson, S. A. Cannistra, *Cancer Res.*, **57**, 1228 (1997).
54. B. F. Haynes *et al.*, *Arthr. Rheum.* **34**, 1434 (1991).
55. S. Katoh, J. B. McCarth, P. W. Kincade, *J. Immunol.* **153**, 3440 (1994).
56. Y. J. Guo *et al.*, *Cancer Lett* **76**, 63 (1994).
57. H. J. Harn *et al.*, *J Clin Gastroenterol.* **22**, 107 (1996).
58. Q. Yu, B.P. Toole, I. Stamenkovic, *J. Exp. Med.* **186**, 1985 (1997)

Bibliography

Conference Abstracts:

Yu, Q., **Moore, R. A.**, Stamenkovic I., and Toole, B. P., (1996) Transfection of malignant murine mammary tumor cells with cDNA for soluble CD44 isoforms prevents ascites tumor formation in vivo. American Society for Cell Biology Annual Meeting. *Mol. Bio. Cell* 7: Supplement

Moore, R. A., Yu, Q., Stamenkovic I., and Toole, B. P., (1997) Transfection of malignant murine mammary tumor cells with cDNA for soluble CD44 isoforms prevents ascites tumor formation in vivo. Proceedings to the Conference for The Department of Defense Breast Cancer Research Program: "*Era of Hope*", Genetics and Biology of Breast Cancer, Vol. II, pp. 695-696.

Publications:

Peterson, R. M., Yu, Q., Stamenkovic, I., and Toole, B. P., (1998) Perturbation of hyaluronan interactions prevents murine mammary ascites tumorigenesis in vivo. (submitted for publication)

Rebecca Moore Peterson is the only individual receiving funds from this effort.