AD_____

GRANT NUMBER: DAMD17-94-J-4121

TITLE: Role of CD44 and Variants in Membrane-Cytoskeleton Interactions, Adhesion, Metastasis and Human Breast Cancers

PRINCIPAL INVESTIGATOR: Lilly Bourguignon, Ph.D.

CONTRACTING ORGANIZATION: University of Miami
Miami, Florida 33136

REPORT DATE: August 1996

TYPE OF REPORT: Annual

DTIC QUALITY INSPECTED 2

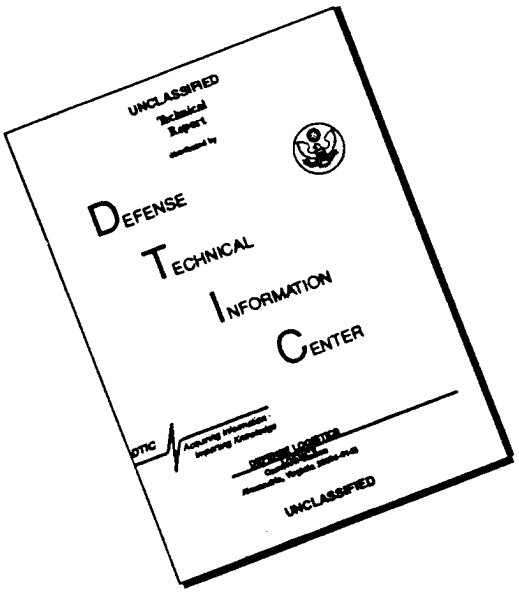
PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

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.

19961022 020

DISCLAIMER NOTICE



THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF PAGES WHICH DO NOT REPRODUCE LEGIBLY.

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	3. REPORT TYPE AND DATES COVERED
	August 1996	Annual (1 Aug 95 - 31 Jul 96)

4. TITLE AND SUBTITLE
Role of CD44 and Variants in Membrane-Cytoskeleton Interactions, Adhesion, Metastasis and Human Breast Cancers

5. FUNDING NUMBERS
DAMD17-94-J-4121

6. AUTHOR(S)
Lilly Bourguignon, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Miami
Miami, Florida 33136

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 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*

CD44 is the major hyaluronan cell surface receptor, and functions as an adhesion molecule in many different cell types including human breast epithelial cells. The coexpression of certain CD44 variants (CD44v), such as CD44v (v10/ex14), with CD44s (standard form) appears to be closely associated with human breast tumor metastasis. In this study we have established a stable transfection of CD44v (v10/ex14) cDNA into normal human breast epithelial cells (HBL100) which contain endogenous CD44s. Our results indicate that coexpression of both CD44v (v10/ex14) and CD44s alters the following important biological properties of these cells: (a) there is a significant reduction in hyaluronic acid (HA)-mediated cell adhesion; (b) there is an increased migration capability in collagen-matrix gel; and (c) these cells constitutively produce certain angiogenic factors which effectively promote tumorigenesis in athymic nude mice. These findings suggest that coexpression of CD44v (v10/ex14) and CD44s may trigger the onset of cell transformation required for breast cancer development.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	48
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500
Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

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

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

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

L.B 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.

L.B 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).

L.B For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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

L.B In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

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

Lilly Y.W. Bourgeois 7/30/96

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

The transmembrane glycoprotein CD44 belongs to a family of cell adhesion molecules which are expressed in a variety of cell types including lymphocytes, myeloid cells, fibroblasts, epithelial cells, endothelial cell, smooth muscle cells, retinal cells, and a subset of glial cells in the central nervous system. (1-5, for recent reviews see 6 and 7). CD44 and its related proteins (CD44 isoforms) display enormous heterogeneity in their primary structures due to either alternative exon splicing and/or post-translational modifications (1-8). It is believed that CD44's structural complexities may be critically important for performing its diverse functions [e.g. regulating signal transduction, cell-cell interactions and cell-extracellular matrix (ECM) interactions] (6,9).

In humans CD44 is encoded by a single gene on chromosome 11 consisting of 19 exons (8). Nine of these exons are capable of undergoing unique splicing events (8,10). Most often, alternative splicing occurs between exons 5 and 15 leading to an insertion of one or more variant exons (v2-v10, or exons 6 through 14) within the membrane proximal region of the extracellular domain (8). The variable primary amino acid sequence of different CD44 isoforms is further modified by extensive N-and O-glycosylations and glycosaminoglycan (GAG) additions (11,12). The extracellular domain of CD44 is known to bind extracellular matrix (ECM)

components such as hyaluronic acid (HA) (13,14) and certain proteoglycans with the chondroitin sulfate modifications (15,16). Other extracellular matrix components, such as fibronectin (17), collagen types I and VI (18,19), also bind to CD44. Furthermore, it is now known that the cytoplasmic domain of CD44 binds to the cytoskeletal protein, ankyrin (20-23). Various modifications, including fatty acylation (24), protein kinase C-mediated phosphorylation (25) and GTP-binding (26), are known to promote the interaction between CD44 and ankyrin (24-26).

Certain CD44 variant (CD44v) isoforms appear to be expressed at high levels on the surface of tumor cells during tumorigenesis and metastasis (27,28). Moreover, CD44v has been shown to be closely involved in the onset of tumor development and metastasis (10, 30-39). For example, one of the CD44v isoforms such as CD44v6 has been shown to confer metastatic behavior on rat pancreatic cells in a spontaneous metastasis assay (29); This molecule is also associated with a poor prognosis in human colon carcinomas (30) and non-Hodgkin's lymphoma (31). Nevertheless, the correlation between CD44v6 expression and its prognostic value for the survival in human breast cancer patients is still very controversial (32-34). In fact, some studies suggest that CD44v6 is merely an epithelial cell differentiation marker; and the usefulness of this molecule as a specific breast tumor cell marker requires further investigation (34). Recently, we have found that the CD44v (v10/ex14) isoform (also called CD44R2) (40) [single insertion of exon 14 (þ62 a.a.)

within the CD44s form] is the only CD44 variant isoform consistently detected in several human cancer tissues including breast carcinomas cells (33-36). CD44v (v10/ex14) contains extensive O- and N-linked glycosylation sites plus chondroitin sulfate (41). Furthermore, this molecule has been shown to be specifically associated with transformed hemopoietic cells (40) and endothelial cells (42). However, at the present time, there is very limited information concerning the mechanism by which CD44v isoform(s), such as CD44v (v10/ex14), may trigger the onset of the tumorigenesis and/or metastasis.

In this study we have cloned CD44v (v10/ex14) cDNA into a pRc/CMV vector and transfected this cDNA into normal human breast epithelial cells (HBL100) containing endogenous CD44s. We have determined that the coexpression of both CD44v (v10/ex14) and CD44s alters several important cellular properties (e.g. adhesion, migration, angiogenic factor production and tumorigenic capability) of the normal mammary epithelial cells. Our results suggest that coexpression of CD44v (v10/ex14) and CD44s may be required for mammary epithelial cell transformation.

BODY (RESULTS):

(A) Transfection and Expression of CD44v10(ex14) In Human Mammary Epithelial Cells Containing CD44s:

In human CD44, alternative splicing often occurs between exons 5 and 15 leading to an insertion of one or more variant exons (v2-v10, or exons 6 through 14) within the membrane proximal region of the extracellular domain (Fig. 1A-a). Recent studies indicate that certain CD44 variant (CD44v) isoforms, such as CD44v (v10/ex14) (Fig. 1A-b), are coexpressed with CD44s in human breast carcinomas cells detected in both early tumor formation and the advanced stages of metastatic human breast cancers (33-36). Therefore, coexpression of these two CD44 isoforms in tumor cells may be required for the progression of human breast cancers (32-39).

In order to test directly whether coexpression of these two CD44 isoforms is involved in mammary epithelial cell transformation, we have established a new, stable transfectant of mammary epithelial cell line which is capable of expressing both CD44v (v10/ex14) and CD44s on its cell surface. Specifically, we have cloned CD44v (v10/ex14) cDNA into a pRc/CMV vector and expressed this cDNA in normal, non-tumorigenic human breast epithelial cells (HBL100) containing CD44s. Using surface iodination and anti-CD44-mediated immunoprecipitation techniques, we have found that both the parental (untransfected) cells and control cells (transfected with vector only) contain only one CD44-

related polypeptide (M.W. þ 95kDa) on the cell surface (Fig.1B, Lane 1 and Lane 2). This 95 kDa protein represents the CD44 standard form (CD44s) as shown previously in many cell types (6). In cells transfected with CD44v (v10/ex14) cDNA, two CD44-related polypeptides (M.W. þ95 kDa and þ116 kDa) are coexpressed on the cell surface (Fig. 1B, lane 3). The 95 kDa protein represents the endogenous CD44s as described previously in other cell types (13,41,46). We believe that the 116 kDa protein belongs to the CD44v isoform encoded by the variant exon 14 (v10) since a similar size of protein (116 kDa) was also detected in other cell lines transfected with CD44v (v10/ex14) cDNA previously (41,46). Using amino acid composition analysis and nucleotide sequence data, we have now verified that 116 kDa protein contains the variant exon 14(v10) structure (42). Thus, the availability of these two cell lines allows us to examine the effects of coexpression of CD44v (v10/ex14) and CD44s on mammary epithelial cell properties (e.g. cell adhesion, invasion, angiogenic factor production and tumorigenesis) as described below.

Hyaluronic Acid (HA)-Mediated Cell Adhesion By Mammary Epithelial Cells Coexpressing Both CD44v (v/10/ex14) and CD44s:

CD44 is the major hyaluronan cell surface receptor, and one of the cellular adhesion molecules in many different cell types (6,7,9). Recently, specific hyaluronic acid (HA) binding motifs have been identified and localized in the extracellular domain of

CD44 isoforms (47). Although all CD44v isoforms contain similar HA binding motifs, certain CD44v isoforms have been reported to display significantly less HA binding than CD44s (41,46). In this study we have compared the HA-mediated cell adhesion properties of parental cells/vector-transfected control cells (expressing CD44s alone) to that of CD44v (v10/ex14)-transfected cells [coexpressing both CD44s and CD44v (v10/ex14)]. Our results clearly indicate that both parental (untransfected) cells and vector-transfected control cells display a high level of CD44-specific cell adhesion to HA-coated plates (Table 1). In contrast, the CD44v (v10/ex14) transfectant cells display greatly reduced CD44-specific cell adhesion to HA plates (Table 1). These findings suggest that (a) CD44s is the major hyaluronan receptor required for HA-mediated cell adhesion in human mammary epithelial cells; and (b) coexpression of both CD44v (v10/ex14) and CD44s either blocks some available sites or changes the binding affinity for HA-mediated cell adhesion.

Analysis of Cell Invasion Properties of Mammary Epithelial Cells Coexpressed With CD44v (v10/ex14) and CD44s:

One of the biological properties commonly associated with cell transformation and tumor development is the ability of tumor cells to penetrate across (or invade) extracellular matrix (ECM) layers (48). In this part of the study we have used an *in vitro* invasion assay which involves growing the mammary epithelial cells [e.g.

CD44v (v10/ex14)-transfectant cells, parental (untransfected) or vector-transfected cells] on the surface (the top layer) of the collagen-gel matrix (Fig. 2 A and B). This cell invasion assay was assessed as the formation of protrusions into the collagen gel using confocal scanning laser microscopy. A series sections (from the bottom to the top of the collagen-gel layers) were collected (at least 0.19pm thick/section) for analysis. The results reveal the presence of numerous CD44-associated cellular protrusions at both the bottom layers (Fig. 2A-frames 1-6) and the top layers of the collagen-gel matrix (Fig. 2A-frames 7-9). In contrast, the plasma membrane of the parental (untransfected) (Fig. 2B) or vector-transfected (data not shown) cells appears attached to the surface (or the top layer) of the collagen-gel matrix (Fig. 2B-frames 7-9) with little detectable CD44-associated cellular protrusions into the bottom layer of the collagen-gel matrix (Fig. 2B, frames 1-5). These data suggest that coexpression of CD44v (v10/ex14) and CD44s reduces HA-mediated cell adhesion and promotes extracellular matrix invasion.

Tumorigenic Assay and Angiogenic Factor Production of Mammary Epithelial Cells Coexpressed With CD44v (v/10/ex14) and CD44s:

In order to determine whether coexpression of CD44v (v10/ex14) and CD44s affects tumorigenic potential, CD44v (v10/ex14) transfectant, parental (untransfected) and vector-transfected cells were separately injected into athymic female nude mice. We then

monitored the ability of each of these cell types to induce subcutaneous tumor formation. Our results indicate that no tumors were formed in those mice injected with either parental (untransfected) or vector-transfected cells (Table 2). However, approximately 60% of the mice injected with CD44v (v10/ex14) transfectant cells develope subcutaneous tumors (Table 2).

These tumors were subsequently biopsied and analyzed for the expression of CD44 by immunoperoxidase staining and RT in situ PCR technique. As shown in Fig. 3A, CD44 immunoreactive staining is primarily observed in the periphery of tumor tissues. We have also used RT in situ PCR and confocal microscopy to identify CD44v (ex14/V10)-containing cells in these tumors. CD44v (ex14/v10) transcripts were specifically detected by using a left primer designed to anneal at the exon 5 and 14 junction sequences. As shown in Fig. 3B, CD44v (v10/ex14) mRNA is present primarily in the peripheral region of the tumor which corresponds to the CD44 immunoperoxidase staining pattern (Fig. 3A). We believe that the RT in situ PCR labeling is specific since no signal is observed when reverse-transcriptase is eliminated from the labeling reactions (Fig. 3C).

It is well known that angiogenesis is required for tumor growth (49,50). In this regard, it is now clear that a multitude of angiogenic and angiostatic factors are involved in the regulation of tumor cell growth (49-53). One particular angiogenic factor by itself may not exert sufficient potency to stimulate or

maintain tumor growth (53). Consequently, it has been suggested that an "angiogenic factor network" may be required for the progression of tumor growth and metastasis (53). In this study two well-characterized angiogenic factors such as basic FGF (51) and IL-8 (52,53) were assayed for their presence in the conditioned media of CD44v (v10/ex14)-transfected and/or parental cells. Our data indicate that CD44v (v10/ex14)-transfectants produce a higher level of basic FGF (Fig. 4A) and IL-8 (Fig. 4B) than parental (untransfected)/vector-transfected cells (Fig. 4 A, B and C). These findings suggest that coexpression of CD44v (v10/ex14) with CD44s in human mammary epithelial cells will promote angiogenesis. Studies on the molecular interactions occurring between CD44v (v10/ex14) and CD44s during signal transduction, cell transformation and tumorigenesis are currently underway.

CONCLUSION:

Hyaluronic acid (HA) is one of the important components of the extracellular matrix (ECM). It is known to cause cell aggregation of a number of different cell types (54); and has been implicated in the stimulation of cell proliferation (55), cell migration (56), cell adhesion (14,57,58) and angiogenesis (59). CD44 is now known to be one of the major HA cell surface receptors (13). High levels of HA production and overexpression of HA receptors (e.g. CD44 isoforms) have been found to be closely associated with tumorigenesis (60-62) including breast cancers (60,61).

It has been suggested that several regions in the extracellular domain of CD44 containing clusters of conserved basic residues (47,63,64) play an important role in CD44 binding to HA. All CD44v isoforms contain similar HA binding domain(s). However, not all CD44 isoforms constitutively bind HA. Often, certain CD44v isoforms display significantly less HA binding than CD44s (65). Information concerning the regulatory factors or mechanisms responsible for the reduction of HA binding by various CD44v isoforms is currently not available.

In this study we have demonstrated that normal mammary epithelial cells containing CD44s alone are capable of adhering to HA-coated plates (Table 1). However, cells coexpressing both CD44v (ex14/v10) and CD44s display a significant reduction in HA-mediated cell adhesion (Table 1). These data indicate that interactions

between CD44v (ex14/v10) and CD44s may have altered the HA binding sites on the cell surface of the human mammary epithelial cells. Previously, CD44v (v10/ex14) (116 kDa protein) has been found to contain O-/N-linked glycosylation sites and chondroitin sulfate additions (41). Therefore, it is possible that post-translational modifications of v10(ex14)-encoded structure induce surface rearrangements or conformational changes in the HA-binding domains resulting in a loss of HA binding. In addition, it is possible that coexpression of CD44v (v10/ex14) and CD44s could prevent the dimerization (clustering) of CD44s molecules required for high affinity binding to HA as suggested by previous studies (6, 66). The reduction of HA-mediated cell adhesion in cells expressing both CD44v (ex14/v10) and CD44s may be one of the earliest events in the onset of tumor migration and invasion processes.

The cytoplasmic domain of CD44 (a portion of the carboxyl terminus including exons 17 and 19, þ70 a.a. long) is highly conserved (þ90%) in most of CD44 isoforms in mammalian cells (23). This domain is important because it provides binding sites for certain cytoskeletal proteins such as ankyrin (19). Cytoskeletal structures such as microfilaments, microtubules and intermediate filaments are known to be involved in a number of important biological activities including cell migration (67), secretion (68) and proliferation (69). In this paper we have shown that CD44v (v10/ex14)-transfectants (a) display a higher migration/invasion capability (Fig. 2A), (b) produce a higher level of certain

angiogenic factors (Fig. 4 B and C); and (c) exhibit more potent tumorigenic potential (Table 2) than either parental (untransfected) or vector-transfected cells (Fig. 4 B and C; and Table 2). It is possible that the unique structure of CD44 (v10/ex14) (e.g. O-/N-linked glycosylation or chondroitin sulfate addition) may cause a constitutive activation of CD44 isoform-cytoskeleton interactions which induce tumor cell migration and invasion. Most recently, Droll et al. have shown that the chondroitin sulfate moiety attached to the v10/ex14 structure of CD44 is capable of binding to other CD44 molecules (66). This unique adhesive interaction may be critically important for (a) homotypic and/or heterotypic cell-cell adhesion in vitro; (b) microemboli formation; and/or (c) the binding between circulating tumor cells and the vascular endothelium in vivo resulting in angiogenic factor production and tumor growth. These hypotheses are currently being tested in our laboratory.

In conclusion, we feel that introduction of CD44v (ex14/v10) into normal mammary epithelial cells expressing CD44s has helped elucidate the role of this particular CD44v isoform during tumor development. Future studies to define the structural and functional relationships between other CD44v isoforms and tumor cell properties are needed in order to provide insights into regulatory mechanisms involved in cell transformation and tumor metastasis during human breast cancers.

FUTURE WORK:

In the coming year, we plan to investigate the physiological role of CD44v3, one of the most important CD44 variants detected in human breast cancer metastasis. Specifically, we plan to carry out the following experiments:

A: To elucidate the CD44v3-cytoskeleton interaction with special emphasis on the structural association between CD44v3 and the cytoskeleton (e.g. actin binding proteins and actin-myosin complexes).

B: To construct CD44v3 deletion or site-directed mutants lacking specific functional domains for cytoskeleton binding. CD44v3 mutant polypeptides will then be expressed in non-metastatic human breast epithelial cells and their structural changes will be correlated with tumor cell motility, invasiveness, and metastasis.

C: To examine human breast tissue samples for CD44v3 expression and correlate qualitative and quantitative levels with the invasive and/or metastatic phenotype during breast cancer progression. This will allow us to establish CD44v3 as a useful metastatic marker for breast cancer detection and prognosis.

A: To elucidate the CD44v3-cytoskeleton interaction with special emphasis on the structural association between CD44v3 and the cytoskeleton (e.g. actin binding proteins and actin-myosin complexes).

(a) Demonstration of A Structural Association between CD44v3 and Cytoskeletal Proteins by Triton X-114 Extraction

Our previous data indicate that a non-ionic detergent extraction procedure involving the use of Triton X-114 detergent plus a temperature-dependent phase separation appears to be more effective than Triton X-100 for isolating cytoskeleton-associated complexes (16-18). In this study Triton X-114 (1% v/v) will be added to the cell samples at 0° (detergent clear point), warmed to 30° (above detergent cloud point) and centrifuged in order to separate the hydrophilic and hydrophobic phases. Predominantly hydrophobic proteins (integral membrane proteins without cytoskeleton attachment) are partitioned into the lower, detergent-rich phase and hydrophilic proteins (peripheral cytoskeletal proteins and cellular proteins with cytoskeleton attachment) are partitioned into the upper, detergent-poor, "aqueous" phase. During Triton X-114 extraction of breast tumor cells with high metastatic potential (Met-1), we have found that CD44v3 is preferentially partitioned into the upper "aqueous" phase (presumably through attachment with peripheral cytoskeletal proteins). Almost all the membrane proteins (but not CD44v3) partition into the lower,

detergent-rich phase. Subsequently, we plan to isolate CD44v3-cytoskeleton complexes from Triton X-114 "aqueous" phase by anti-CD44v3-mediated immunoprecipitation. Those cytoskeletal protein(s) linked to CD44v3 will be identified by immunoblotting with specific cytoskeletal protein antibody (e.g. actin, myosin, fodrin, ankyrin, 4.1 protein, talin and vinculin, etc.).

(b) Binding of ^{125}I-Cytoskeletal Protein to CD44v3-Conjugated Beads

In this study CD44v3 will be isolated by rabbit anti-CD44v3 conjugated Sepharose beads. CD44v3-conjugated beads will then be incubated with ^{125}I-labeled cytoskeletal proteins (e.g. actin, myosin, fodrin, ankyrin, 4.1 protein, talin and vinculin) for 2h at 4°. The CD44v3-cytoskeleton complexes will be washed extensively. Non-specific binding (determined by incubating samples with at least 100-fold excess unlabeled cytoskeletal proteins) will be subtracted from experimental values in order to determine the specific binding which occurs between CD44v3 and certain cytoskeletal protein(s).

In order to further confirm the cytoskeleton binding domain of CD44v3 and demonstrate the role of the cytoskeleton in the regulation of CD44v3's function, we plan to use a molecular biological approach which involves the use of <u>in vitro</u> mutagenesis and expression in non-metastatic breast epithelial cells as described below.

Anticipated Results:

(i) We predict that various cytoskeletal proteins that complex with CD44v3, either directly or indirectly, will be successfully identified.

(ii) We anticipate that the direct interactions between various cytoskeletal proteins and CD44v3 will be further verified by *in vitro* binding studies.

Aim 2: To construct CD44v3 deletion or site-directed mutants lacking specific functional domains for cytoskeleton binding. CD44v3 mutant polypeptides will then be expressed in nonmetastatic human breast epithelial cells and their structural changes will be correlated with tumor cell motility, invasiveness, and metastasis.

The procedures for cloning and expression of CD44v3 in human breast cancer cells are described as follows: The RT-PCR primers (i.e. TACATCAGTCACAGACCTGC and ATCCATGAGTGGTATGGGAC) will be used to permit the direct cloning of the CD44v3-related amplification products into pRc/CMV/CD44 for expression in mammalian cells. The amplification products will contain unique restriction enzyme sites for Hpa I (476) and Tth I(835). The Hpa I site is also unique in the human CD44s insert of pRc/CMV/CD44s (provided by Dr. Eugene Butcher, Stanford University), while there are two sites for Tth I. Therefore, the amplification products can be inserted directly into pRc/CMV/CD44s which has been partially digested with Tth I and completely digested with Hpa I. This will allow the insertion of

the CD44v3 sequences directly into the CD44s coding sequence of pRc/CMV/CD44s at precisely the correct location and reading frame. Final constructs containing full-length CD44v3 structures will be used for the transfection of eukaryotic cells. In addition, we plan to create deletion and/or site-directed mutants that lack particular sequences for ankyrin-binding in the cytoplasmic domain.

These CD44v3 constructs will then be transfected into a non-invasive human breast cancer cell lines such as MCF10A and HBL100 and characterized functionally by analyzing the effects on CD44v3-cytoskeleton interaction, invasion and metastasis. In vitro characterization of individual CD44v3 deletion mutants will focus on CD44v3-cytoskeleton interaction using the same procedures as described above. Using information obtained from mutational analysis, we will then correlate the expression of functional domains specific for CD44v3-cytoskeleton association with motility and invasive/metastatic behavior in breast cancer cells. Cellular motility will be performed using in vitro invasion assays and "invadopodia" formation analysis as described previously. Tumorigenesis and invasive/metastatic properties of individual transfectants will be evaluated by subcutaneous (or by mammary fat pad or tail vein) injection in 6-week old female nude mice with quantitation by the size and number of tumors formed as well as various sites of metastases as compared to nontransfected controls. Approximately, 7-10 mice per experimental group will be injected with $1x10^7$ cells. After specific CD44v3 domains have been

identified as critical to cytoskeleton binding and/or metastasis, we will further determine key amino acids by site-directed mutagenesis. These may involve conserved residues within known consensus sequences or previously unidentified sequences. Identification of these key regions will provide further clues to mechanistic aspects underlying breast cancer metastasis.

Anticipated Results: We predict that CD44v3's functional domains related to cytoskeletal binding site(s) will be mapped out using both deletion and site-directed mutagenesis methods.

C: To examine human breast tissue samples for CD44v3 expression and correlate qualitative and quantitative levels with the invasive and/or metastatic phenotype during breast cancer progression. This should allow us to establish CD44v3 as a useful metastatic marker for breast cancer detection and prognosis.

The procedures used for generating polyclonal and monoclonal antibodies against CD44v3 will be performed as described previously. Standard antibody characterization methods such as ELISA, immunoblotting, and immunoprecipitation will be used to verify the specificity and titers of polyclonal and monoclonal antibodies. Specifically, breast cancer tissue sections [e.g. node positive vs node negative breast cancer samples; and high- vs low-grade histologies of ductal carcinoma in situ (DCIS)] will be compared. In order to control for other prognostic factors, we will limit the analysis of invasive cancers to tumors of similar size

(i.e. between 1 and 3 cm) and nuclear grade (low to intermediate). The samples will be fixed with 2% paraformaldehyde and processed for standard immunoperoxiase staining using polyclonal or monoclonal anti-CD44v3 antibody. Some samples will also be processed for immunofluorescence staining using a laser scanning confocal microscope (MultiProbe 2001 Invert CLSM System, Molecular Dynamics) for both qualitative and quantitative measurements. The relationship between CD44v3 expression and clinicopathological indices will be analyzed by Fisher's exact t test. Correlation between the fraction of CD44v3 positive tumor cells in normal, non-metastatic and metastatic breast tissues will be assessed by the Spearman rank correlation coefficient as described previously. The results of these experiments will allow us to establish a possible relationship between CD44v3 expression and the progression of human breast cancers.

Anticipated Results:

We anticipate that there will be a strong correlation between the level of CD44v3 expression and invasive/metastatic behavior. Moreover, domains previously identified as critical to cytoskeletal interaction and/or those associated with invasion and metastasis will be found to be upregulated in those breast cancers which involve axillary lymph nodes. In addition, we anticipate a correlation between expression of CD44v3 and DCIS lesion of high histologic grade (i.e., those expected to become invasive).

MATERIALS AND METHODS:

Cell Lines: The HBL100 cell line (43) was obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % fetal calf serum, L-glutamine, penicillin (100 units/ml), and streptomycin (100 m/ml) (GIBCO BRL, Gaithersburg, MD). The cells were maintained in a humidified incubator under a 5 % CO_2 in air atmosphere at 37°C.

Stable Transfection: The human variant CD44 full length cDNA (standard + exon14) was cloned in the pRC/CMV eukaryotic expression vector (Invitrogen, San Diego, CA), a kind gift from Dr. E. C. Butcher (Stanford University, Palo Alto, CA.) which was then transfected into the HBL100 cells using the lipofectAMINE reagent (GIBCO BRL) according to the manufacture's protocol. Three days after transfection, cells were split 1:10 in complete medium containing G418 (GIBCO BRL) at a concentration of 750 µg/ml. Individual colonies were isolated after two weeks; and stable cell lines were established and maintained in the complete medium containing 400 þg/ml G418.

Analysis of CD44 Expression At the Protein Level: Transfected and parental HBL100 cells were surface-iodinated by the lactoperoxidase method (44). Cells were then solubilized and CD44 proteins immunoprecipitated using monoclonal rat anti-CD44 antibody as described previously (21). Immunoprecipitants were analyzed by

polyacrylamide gel (7.5%) electrophoresis and detected by autoradiography.

Cell Adhesion Assays: The 96-well ELISA plate (CORNING Glass Works, Corning, NY) was coated with rooster comb HA (SIGMA Chem. Co., St. Louis, MO) as described previously (22). Briefly, the ELISA plate was incubated with 2 mg/ml rooster comb HA at 22° C for 16 hr, followed by rinsing three times with PBS containing 2 % BSA. Additional washing with PBS were carried out before incubating with labeled cells in the adhesion assay. Cells were metabolically labeled with Trans[^{35}S]methionine (30 þCi/ml, ICN) as described previously (3). After labeling, the cells were washed twice in PBS and detached by incubating in PBS containing 5 mM EDTA at 37°C for 5 min. Radioactively labeled single-cell suspensions (5.0 x 10^{5} cpm) were incubated in individual wells of a HA-coated ELISA plate at 4° C for 60 min. After incubation, the wells were washed three times with PBS. The adherent cells were solubilized in PBS containing 1 % SDS and the bound radioactivity was measured by liquid scintillation counting. Nonspecific binding was determined by including 200 þg/ml soluble HA in the HA-coated wells during the incubation. In some cases, the background level of binding was determined by cell adhesion performed in the presence of monoclonal rat anti-CD44 antibody treatment. The results were expressed in terms of CD44-specific binding in which the background levels of

binding have been subtracted. Data are expressed as mean cpm ± SEM of triplicate determinations.

In Vitro Cell Invasion Assays: Chambered coverglasses (8 chambers/coverglass, Nunc, Inc., Naperville, IL) were coated with rat tail type I collagen gel (150 þl/1.0 cm^2) by incubating at 37°C overnight. The collagen gel was equilibrated with DMEM supplemented with 10% FCS and L-glutamine prior to the use. Trypsinized cells were suspended in the complete media (DMEM, 10% FCS, glutamine) and 10,000 cells per chamber were incubated at 37°C overnight in a humidified 5% CO_2 incubator. Cell surface CD44 molecules were immunostained using monoclonal rat anti-CD44 antibodies as previously described (23). Cell invasion was assessed as the formation of protrusions into the collagen gel using confocal scanning laser microscopy (Molecular Dynamics, Sunnyvale, CA). A series of at least 30 optical sections were collected (0.19þm thick) for analysis.

Tumorigenesis Assays: Each cell line was inoculated subcutaneously into two sites/mouse (either 10^7 or 10^8 cells/site) of female athymic nude mice, and tumor formation was monitored for six months. Tumor mass was estimated using the formula $(d^2 \times D)/2$, where d and D represent the small and large diameter of the tumor, respectively.

Immunohistochemistry and RT in situ PCR: Tumors were analyzed for the expression of CD44 proteins by immunoperoxidase staining of these paraffin-embedded tissues described previously (35). To detect the expression of CD44v (ex14/v10) transcripts in tumors, the RT in situ PCR protocol described by Nuovo (45) was employed with a minor modification. The left PCR primer was designed to anneal at the junction of exon5 and exon14, and the right PCR primer annealed in exon15. The right primer was 5'-ATCCATGAGTGGTATGGGAC-3' and the left primer was 5'-CTGCTACCAATAGGAATGAT-3'. This pair of PCR primers allowed the specific amplification of the human CD44v (ex14/v10) transcripts, but not other CD44 variants, or mouse CD44 transcripts or mouse genomic DNA. In the PCR mixture, we have utilized the direct incorporation of FITC-digoxigenin-11-dUTP, and the PCR was carried out twenty cycles of 55°C for 2 min and 94 C for 1 min. The fluorescent signals were then analyzed by laser confocal microscopy.

Measurement of Angiogenic Factors: Cells were grown in complete medium on 100 mm tissue culture dishes. Cells were then washed three times with serum free DMEM and incubated in 3 ml of SF-DMEM containing 1 mg/ml BSA (type V, Sigma Chemical Co., St. Louis, MO) for an additional 24 hr. at 37°C in a 5% CO2 humidified chamber. The conditioned medium was collected and analyzed for the concentration of angiogenic factors (e.g. basic FGF, and IL-8)

using Quantikine immunoassays according to the manufacture's protocol (R&D system, Inc., Minneapolis, MO).

REFERENCES

1. Haynes, B.F., Telen, M.J., Hale, L.P., and Denning, S.M. CD44-a molecule involved in leukocyte adherence and T-cell activation. Immunol. Today, 10: 423-428, 1989.

2. Picker, L.J., Nakache, M., and Butcher, E.C. Monoclonal antibodies to human lymphocyte homing receptors define a novel class of adhesion molecules on diverse cell type. J. Cell Biol., 109: 927-937, 1989.

3. Bourguignon, L.Y.W., Lokeshwar, V.B., He, X., Chen, X., and Bourguignon, G.J. A CD44-like endothelial cell transmembrane glycoprotein (GP116) interacts with extracellular matrix and ankyrin. Mol. Cell Biol., 12: 4464-4471, 1992.

4. Salmi, M., Gron-Virta, K., Sointu, P., Grenman, R., Kalmo, H., and Jalkanen, S. Regulated expression of exon v6 containing isoforms of CD44 in man: Downregulation during malignant transformation of tumors of squamocellular origin. J. Cell Biol., 122: 431-442, 1993.

5. Chaitin, M.H., Wortham, H.S., and Grun-Zinkernagel, A.M. Immunocytochemical localization of CD44 in the mouse retina. Exp. Eye Res., 58: 359-365, 1994.

6. Lesley, J., Hyman, R., and Kincade, P.W. CD44 and its interaction with extracellular matrix. Advances Immunol. 54: 271-335, 1993.

7. Bourguignon, L.Y.W. Interaction between the membrane-cytoskeleton and CD44 during lymphocyte signal transduction and cell adhesion. Current Topics In Membranes "The plasma membrane-cytoskeleton interface: Template for protein domains, vesicle trafficking, and signal transduction) (ed. Nelson, W. J.) (in press, 1996).

8. Screaton, G.R., Bell, M.V., Jackson, D.G., Cornelis, F.B., Gerth, U., and Bell, J.I. Genomic structure of DNA coding the lymphocyte homing receptor CD44 reveals at least 12 alternative spliced exons. Proc. Natl. Acad. Sci. USA, 89:12160-12164, 1992.

9. Gunthert, U. CD44: a multitude of isoforms with diverse functions. Curr. Topics in Microbiol. and Immunol., 184:47-64, 1993.

10. Herrlich, P., Zollar, M., Pals, S.T., and Ponta, H. CD44 splice variants: metastases meet lymphocytes. Immunol. Today, 14: 395-399, 1993.

11. Brown, T.A., Bouchard, T., St. John, T., Wayner, E., and Carter, W.G. Human keratinocytes express a new CD44 core protein (CD44E) as a heparin-sulfate intrinsic membrane proteoglycan with additional exons. J. Cell Biol., 113:207-221, 1991.

12. Haggerty, J.G., Bretton, R.H., Milstone, L.M. Identification and characterization of a cell surface proteoglycan on keratinocytes. J. Invest. Dermatol., 99:374-380, 1992.

13. Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C.B., and Seed, B. CD44 is the principle cell surface receptor for hyaluronate. Cell, 61: 1303-1313, 1990.

14. Culty, M., Miyake, K., Kincade, P. W., Silorski, E., Butcher, E. C. and Underhill, C. B. (1990) The hyaluronate receptor is a member of the CD44(H-CAM) family of cell surface glycoproteins. J. Cell Biol., 111,2765-2774, 1990.

15. Naujokas, M.F., Morin, M., Anderson, M.S., Peterson, M., and Miller, J. The chondroitin sulfate form of invariant chain can enhance stimulation of T cell responses through interaction with CD44. Cell, 74: 257-268, 1993.

16. Toyama-Sorimachi, N., Sorimachi, H., Tobita, Y., Kitamura, F., Yagita, H., Suzuki, K., and Miyasaka, M. A novel ligand for CD44 is serglycin, a hematopoietic cell lineage-specific proteoglycan. J. Biol. Chem., 270: 7437-7444, 1995.

17. Jalkanen, S.P. and Jalkanen, M. Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin., J. Cell Biol., 116: 817-825, 1992.

18. Wayner, E.A. and Carter, W.G. Transformation-dependent alterations is glycoproteins of extracellular matrix of human fibroblasts. Characterization of GP250 and the collagen-like GP140. J. Biol.Chem., 257:13805-13015, 1982.

19. Carter, W.G. and Wayner, E.A. Characterization of the class III collagen receptor, a phosphorylated transmembrane glycoprotein expressed in nucleated human cells. J. Biol. Chem., 263: 4193-4201, 1988.

20. Kalomiris, E.L. and Bourguignon, L.Y.W. Mouse T-lymphoma cells contain a transmembrane glycoprotein (GP85) which binds ankyrin. J. Cell Biol., 106:319-327, 1988.

21. Lokeshwar, V.B. and Bourguignon, L.Y.W. Post-translational protein modification and expression of ankyrin-binding site(s) in GP85 and its biosynthetic precursors during T-lymphoma membrane biosyntheses. J. Biol. Chem., 266: 17983-17989, 1991.

22. Bourguignon, L.Y.W., Lokeshwar, V.B., Chen, X., and Kerrick, W.G.L. Hyaluronic acid (HA)-induced lymphocyte signal transduction and HA receptor (GP85/CD44)-cytoskeleton interaction. J. Immunol., 151: 6634-6644, 1993.

23. Lokeshwar, V.B., Fregien, N., and Bourguignon, L.Y.W. Ankyrin binding domain of CD44(GP85) is required for the expression of hyaluronic acid-mediated adhesion function. J. Cell Biol., 126: 1099-1109, 1995.

24. Bourguignon, L.Y.W., Kalomiris, E.L., and Lokeshwar, V.B. Acylation of the lymphoma transmembrane glycoprotein, GP85, may be required for GP85-ankyrin interaction. J. Biol. Chem., 266: 11761-11765, 1991.

25. Kalomiris, E.L. and Bourguignon, L.Y.W. Lymphoma protein kinase C is associated with the transmembrane glycoprotein, GP85, and may function in GP85-ankyrin binding. J. Biol. Chem., 264: 8113-8119, 1989.

26. Lokeshwar, V.B. and Bourguignon, L.Y.W. The lymphoma glycoprotein GP85(CD44) is a novel G-protein which regulates GP85(CD44)-ankyrin interaction. J. Biol. Chem., 267:22073-22078, 1992.

27. Matsumura, Y. and Tarin, D. Significance of CD44 gene products for cancer diagnosis and disease evaluation. Lancet, 340:1053-1058, 1992.

28. Tanabe, K.K., Ellis, L.M., and Saya, H. Expression of CD44R1 adhesion molecule in colon carcinoma and metastases. Lancet, 341: 725-726, 1993.

29. Gunthert, U., Hoffman, M., Rudy, W., Reber, S., Zoller, M., Hauþmann, I., Matzku, S., Wenzel, A., Ponta, H., and Herrlich, P. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell, 65: 13-24, 1993.

30. Mulder, J.-W. R., Kruyt, P., Sewnath, M., Oosting, J., Seldenrijk, C.A., Weidema, W.F., Offerhaus, G.F.A., and Pals, S.T. Colorectal cancer prognosis and expression of exon-v6-containing CD44 proteins. Lancet, 344:1470-1472, 1994.

31. Stauder, R., Eisterer, W., Thaler, J., Gunthert, U. CD44 variant isoforms in non-Hodgkinþs lymphoma: a new independent prognostic factor. Blood, 85:2885-2899, 1995.

32. Dall, P., Heider, K.-H., Sinn, H.-P., Skroch-Angel, P., Adolf, G., Kaufmann, M., Herrlich, P., and Ponta, H. Comparison of immunohistochemistry and RT-PCR for detection of CD44v-expression, a new prognostic factor in human breast cancer. Int. J. Cancer, 60:471-477, 1995.

33. Kaufmann, M., Meider, K.-H., Sinn, H.-P., von Minckwitz, G., Ponta, H., and Herrlich, P. CD44 variant exon epitopes in primary breast cancer and length of survival. Lancet, 345:615-619, 1995.

34. Friedrichs, K., Franke, F., Lisboa, B.-W., Kugler, G., Gille, I., Terpe, H.-J., Holzel, F., Maass, H., and Gunthert, U. CD44 isoforms correlate with cellular differentiation but not with prognosis in human breast cancer. Cancer Res., 55:5424-5433, 1995.

35. Iida, N. and Bourguignon, L.Y.W. New CD44 splice variants associated with human breast cancers. J. Cell. Physiol., 162: 127-133, 1995.

36. Bourguignon, L.Y.W., Iida, N., Welsh, C.F., Zhu, D., Krongrad, A., and Pasquale, D. Involvement of CD44 and its variant isoforms in membrane-cytoskeleton interaction, cell adhesion and tumor metastasis. J. Neuro-Oncol., 26:201-208, 1995.

37. Joensuu, H., Klemi, P.J., Toikkane, S., and Jalkanen, S. Glycoprotein CD44 expression and its association with survival in breast cancer. Am. J. of Pathol., 143:867-874, 1993.

38. Rodriguez, C., Monges, G., Rouanet, P., Dutrillaux, B., Lefrancois, D., and Theillet, C. CD44 expression patterns in breast and colon tumors: A PCR-based study of splice variants. Int. J. Cancer, 64:347-354, 1995.

39. Culty, M., Shizari, M., Thompson, E.W., and Underhill, C.B. Binding and degradation of hyaluronan by human breast cancer cell lines expressing different forms of CD44: correlation with invasive potential. J. Cell. Physiol., 160:275-286, 1994.

40. Dougherty, G.J., Lansdorp, P.M., Cooper, D.L., and Humphries, R.K. Molecular cloning of CD44R1 and CD44R2, two novel isoforms of the human CD44 lymphocyte "homing" receptor expressed by hemopoietic cells. J. Exp. Med., 174: 1-5, 1991.

41. Jackson, D.G., Bell, J.I., Dickinson, R., Timans, J., Shields, J., and Whittle, N. Proteoglycan forms of the lymphocyte homing receptor CD44 are alternatively spliced variants containing the v3 exon. J. Cell Biol., 128: 673-685, 1995.

42. Lokeshwar, V.B., Iida, N. and Bourguignon, L.Y.W. The cell adhesion molecule, GP116, is a new CD44 variant (ex14/v10) involved in hyaluronic acid binding and endothelial cell proliferation (submitted to J. Biol. Chem.) 1996.

43. Gaffney, E.V. A cell line (HBL100) established from human breast milk. Cell Tissue Res., 227: 563-568, 1982.

44. Morrison, M. Lactoperoxidase-catalyzed iodination as a tool for investigation of proteins. Methods in Enzymol., 70:214-220, 1980.

45. Nuove, G.J. RT in situ PCR with direct incorporation of digoxigenin-11-dUTP: Protocol and applications. Biochemica, 11: 4-6, 1994.

46. van der Voort, R., Manten-Horst, E., Smit, L., Ostermann, E., van den Gerg, F., and Pals, S. T. Binding of cell-surface expressed CD44 to hyaluronate is dependent on splicing and cell type. Biochem. Biophy. Res. Commun., 214:137-144, 1995.

47. Yang, B., Yang, B.L., Savani, R.C., and Turley, E.A. Identification of a common hyaluronan binding motif in the hyaluronan binding proteins RHAMM, CD44, and link protein. EMBO J., 13: 286-296, 1994.

48. Liotta, L.A., Steeg, P.S., Stetler-Stevenson, W.G. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell, 64:327-336, 1991.

49. Folkman, J. Tumor angiogenesis. Adv. Cancer Res., 43:175-203, 1985.

50. Dvorak, H.F., Brown, .L.F., Detmar, M., and Dvorak, A.M. Review: vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am. J. Pathol., 146:1029-1039, 1995.

51. Folkman, J., and Klagsbrun, M. Angiogenic factors. Science, 235:442-447, 1987.

52. Kock, A.E., Polverini, P.J., Kunkel, S.L., Harlow, L.A., DiPietro, L.A., Elner, V.M., Elner, S.G., and Strieter, R.M. Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science, 258:1798-1801, 1992.

53. Strieter, R.M., Polverini, P.J., Arenberg, D.A., Walz, A., Opdenakker, G., Van Damme, J., and Kunkel, S.L. Role of C-X-C chemokines as regulators of angiogenesis in lung cancer. J. Leukoc. Biol., 57:752-762, 1995.

54. Green, S.J., Tarone, G., and Underhill, C.B. Aggregation of macrophages and fibroblasts is inhibited by a monoclonal antibody to the hyaluronate receptor. Exp. Cell Res., 178:224-232, 1988.

55. West, D.C. and Kumar, S. The effect of hyaluronate and its oligosaccharides on endothelial cell proliferation and monolayer integrity. Exp. Cell Res., 183:179-196, 1989.

56. Turley, E.A., Austen, L., Vandeligt, K., and Clary, C. Hyaluronan and a cell-associated hyaluronan binding protein regulate the locomotion of ras-transformed cells. J. Cell Biol., 112:1041-1047, 1991.

57. Miyake, K., and Kincade, P.W. A new cell adhesion mechanism involving hyaluronate and CD44. Curr. Top. Microbiol. Immunol., 166:87-90, 1990.

58. Miyake, K., Underhill, C.B., Lesley, J., and Kincade, P.W. Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. J. Exp. Med., 172:69-75, 1990.

59. Rooney, P., Kumar, S., and Wang, M. The role of hyaluronan in tumor neovascularization. Int. J. Cancer, 60:632-636, 1995.

60. Bertrand, P., Girard, N., Delpech, B., Duval, C., DþAnjou, J., and Dauce, J.P. Hyaluronan (hyaluronic acid) and hyaluronectin in the extracellular matrix of human breast carcinomas: comparison between invasive and non-invasive areas. Int. J. Cancer, 52:1-6, 1992.

61. de la Tore, M., Wells, A.F., Bergh, J., and Lindgren, A. Localization of hyaluronan in normal breast tissue, radical scar, and tubular breast carcinoma. Human Pathol., 24, 1294-1297, 1993.

62. Zhu, D. and Bourguignon, L.Y.W. Overexpression of CD44 in $p185^{neu}$-transfected NIH3T3 cells promotes an up-regulation of hyaluronic acid-mediated membrane-cytoskeleton interaction and cell adhesion. Oncogene, (in press, June 1996).

63. Peach, R.J., Hollenbaugh, D., Stamenkovic, I., and Aruffo, A. Identification of hyaluronic acid binding sites in the extracellular domain of CD44. J. Cell Biol., 122:257-264, 1993.

64. Liao, H.X., Lee, D.M., and Haynes, B.F. N-terminal and central regions of the human CD44 extracellular domain participated in cell surface hyaluronan binding. J. Immunol., 155:3938-3945, 1995.

65. Stamenkovic, I., Amiot, M., Pesando, J.M., and Seed, B. The hemopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronon-bearing cells. EMBO J., 10:343-347, 1991.

66. Droll, A., Doughery, S.T., Chiu, R.D., Dirks, J.F., Mcbride, W.H., Cooper, D.L., Dougherty, G.J. Adhesive interactions between alternatively spliced CD44 isoforms. J. Biol. Chem., 270: 11567-11573, 1995.

67. Satterwhite, L.L. and Polland, T.D. Cytokinesis. Curr. Opin. Cell. Biol., 4:43-52, 1992.

68. Trifaro, J.-M., and Vitale, M.L. Cytoskeleton dynamics during neurotransmitter release. Trends in Neurosci., 16:466-472, 1993.

69. Brackman, D., Trydal, T., Lillehayg, J.R. and Aarskog, D. Reorganization of the cytoskeleton and morphological changes induced by 1,25-dihydroxyvitamin D3 in C3H/10T/2 mouse embryo fibroblasts: relation to inhibition of proliferation. Exp. Cell Res., 201:485-493, 1992.

APPENDIX

Table 1: CD44-mediated adhesion of metabolically labeled cells to HA-coated plates.

Cell Lines[a]	CD44-Specific Cell Adhesion[b]	% of Control
Parental HBL100	130,000 ± 14,000	100
Neo-transfected HBL100	125,000 ± 11,000	97
CD44v(v10/ex14)-transfected HBL100	N.D.[c]	0

---

[a]Tran^{35}S-labeled cells [e.g. parental HBL100, neo-transfected HBL100 and CD44v(v10/ex14)-transfected HBL100] were pretreated with or without monoclonal rat anti-CD44 antibody. Subsequently, these cells (5.0×10^5 cpm) were incubated in tissue culture wells coated with hyaluronic acid (HA) as described in the Materials and Methods. The background level of binding was determined by performing cell adhesion in the presence of monoclonal rat anti-CD44 monoclonal antibody treatment.

[b]The results expressed in terms of CD44-specific binding have the background levels of binding subtracted. Data are expressed as mean cpm ± SEM of triplicate determinations.

[c]N.D., not detectable.

Table 2: Tumorigenicity assay for HBL100 cell lines [parental HBL100, vector-transfected HBL100, CD44v (ex14/v10) cDNA-transfected HBL100 cells] in athymic nude mice.

Cell Lines[a]	Tumor Frequency (no. of tumor bearing mice/no. of total mice used)
Parental HBL100	0/4
Vector-transfected HBL100	0/4
CD44v(v10/ex14)-transfected HBL100	5/8[b]

[a]Each cell line was inoculated subcutaneously into two sites/mouse (either 10^7 or 10^8 cells/site) of female athymic nude mice, and tumor formation was monitored for six months.

[b]Tumor nodules (an average cross-sectional area of 3.8± 0.6 mm^2) were detected in athymic nude mice at various areas close to the original cell inoculation sites.

FIGURE LEGENDS

Fig. 1: Exon map of human CD44 gene and analysis of surface CD44 expression in various cell lines [e.g. parental HBL100, vector-transfected HBL100, CD44v (ex14/v10) cDNA-transfected HBL100 cells].

A-a: All 9 possible exon insertions (e.g. exons 6-14) between exon 5 and exon 15 of human CD44 gene.

A-b: Exon 14 is inserted between exon 5 and exon 15 [designated as CD44v (v10/ex14)].

B: Analysis of surface CD44 expression by monoclonal rat anti-CD44-mediated immunoprecipitation from surface ^{125}I-labeled cells.

Lane 1: Autoradiogram of CD44s (þ95 kDa) immunoprecipitated from surface ^{125}I-labeled parental HBL100 cells by rat anti-CD44 antibody.

Lane 2: Autoradiogram of CD44s (þ95 kDa) immunoprecipitated from surface ^{125}I-labeled vector-transfected stable HBL100 cells by rat anti-CD44 antibody.

Lane 3: Autoradiogram of both CD44s (þ95 kDa) and CD44v (ex14/v10) (þ116 kDa) immunoprecipitated from surface ^{125}I-labeled CD44v (ex14/v10)-transfected stable cells.

Fig. 2: Cell invasion by mammary epithelial cells coexpressing CD44v (v10/ex14) and CD44s.

Cells [CD44v (ex14/v10)-transfected cells (A) and parental HBL100 cells (B)] were grown on coverglasses coated with rat tail

type I collagen gel (150 þl/1.0 cm^2) followed by immunofluorescence staining with monoclonal rat anti-CD44 antibody as described in the Materials and Methods. Cell invasion was assessed as the formation of protrusions into the collagen gel using confocal scanning laser microscopy. A series of at least 30 optical sections along the z-axis from the bottom of the gel to the top of the gel were collected (0.19þm thick) for analysis. Frames 1-5 represent the bottom layer of the collagen-gel matrix and frames 6-9 represent the top layer of the collagen-gel matrix. [Arrow heads indicate the presence of invasive protrusions].

Fig. 3: Immunohistochemical staining and RT in situ PCR analysis of tumors obtained from athymic mice injected with CD44v (ex14/v10) cDNA-transfected HBL100 cells.

A: Tumor tissues obtained from athymic mice injected with CD44v (ex14/v10) cDNA-transfected HBL100 cells were processed for immunoperoxidase staining of CD44 using monoclonal rat anti-CD44 antibody.

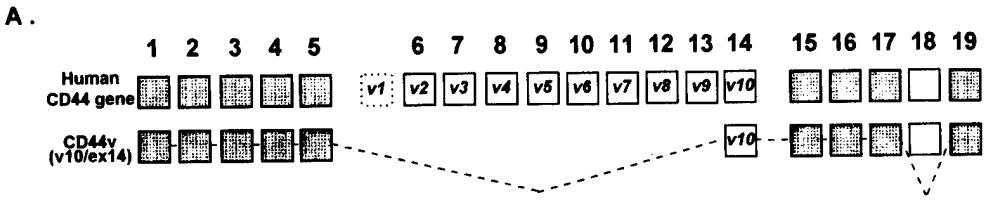
B: Detection of CD44v (ex14/v10) specific transcripts by RT in situ PCR using specific primers (5'-ATCCATGAGTGGTATGGGAC-3' and 5'-CTGCTACCAATAGGAATGAT-3') and FITC-digoxigenin-11-dUTP as described in the Materials and Methods.

C: In RT in situ PCR control samples, reverse-transcriptase was eliminated from the labeling reactions. Note that no signal was detected in these samples. The scale bar represents 20µm.

Fig. 4: Measurement of angiogenic factors in both parental HBL100 and CD44v (ex14/v10) cDNA-transfected HBL100 cells.

Cells [either parental HBL100 or CD44v (ex14/v10) cDNA-transfected HBL100 cells] were grown in the complete growth medium, and the conditioned medium from these two cell lines was analyzed for the concentration of angiogenic factors, such as basic FGF (A) and IL-8 (B), using Quantikine immunoassays as described in Material and Methods. Statistical analyses indicate that the increase of bFGF (A) and IL-8 (B) production in CD44v (ex14/v10) cDNA-transfected cells was significantly greater than in the parental HBL100 cells [with $p \geq 0.06$ and $p \geq 0.0008$, respectively].

Fig. 1


A.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Human CD44 gene
v1 v2 v3 v4 v5 v6 v7 v8 v9 v10
CD44v (v10/ex14)
v10

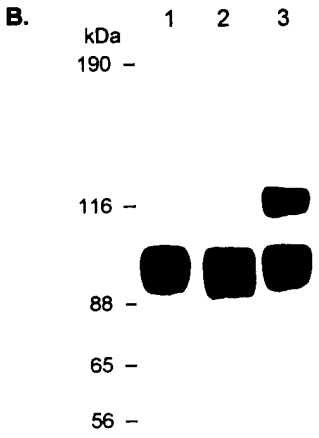
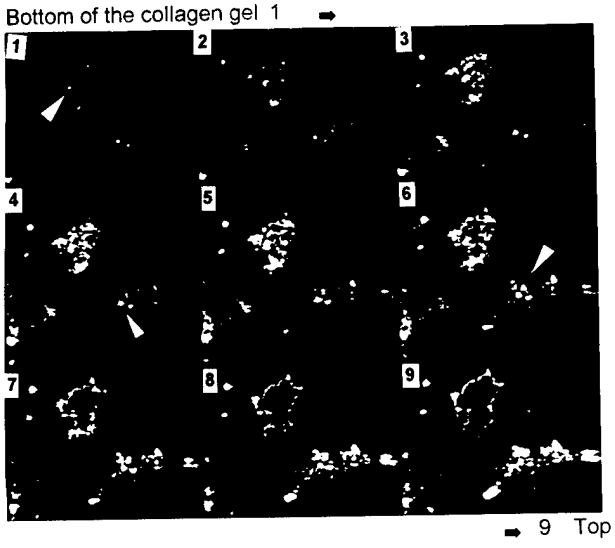

B.
kDa
1 2 3
190 –
116 –
88 –
65 –
56 –

Fig. 2

A.


Bottom of the collagen gel 1
1
2
3
4
5
6
7
8
9
9 Top

B.

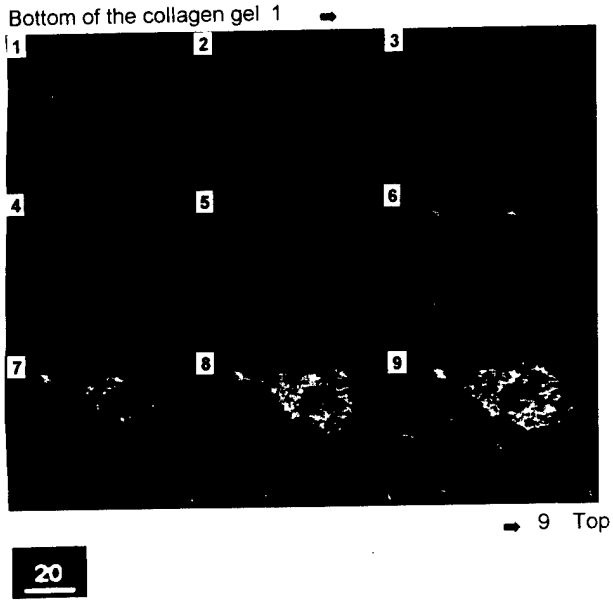

Bottom of the collagen gel 1
1
2
3
4
5
6
7
8
9
9 Top
20

Fig. 3

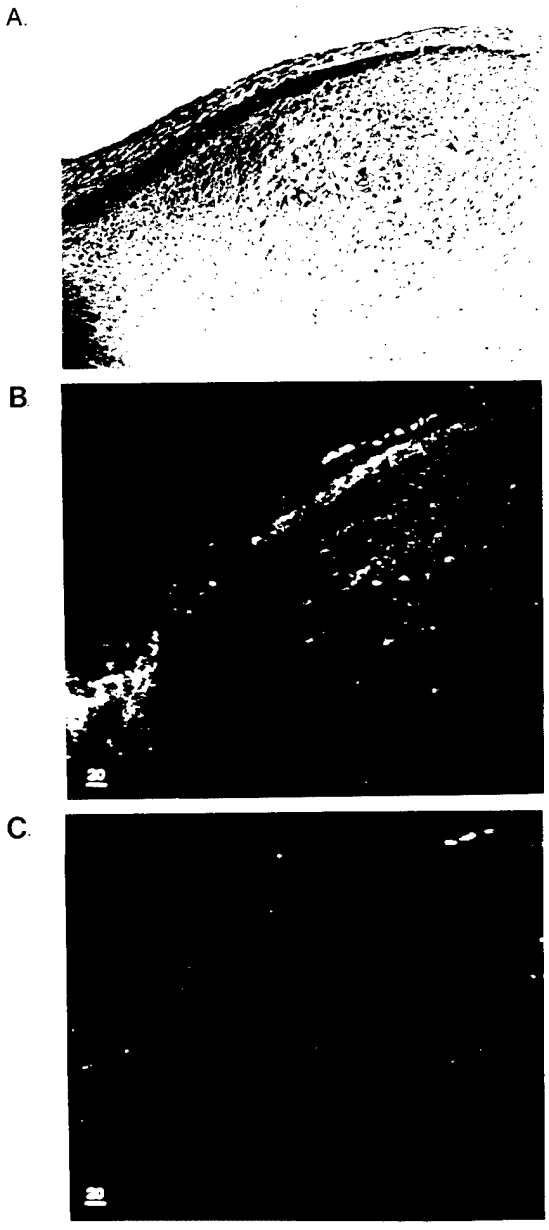
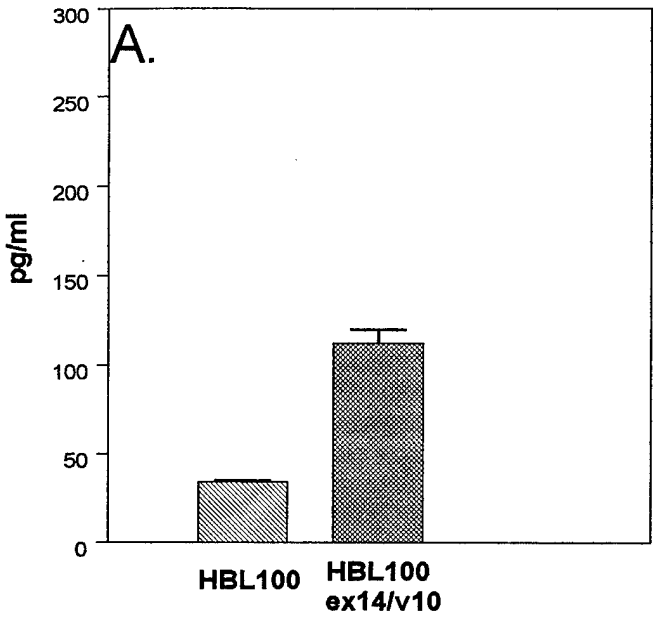
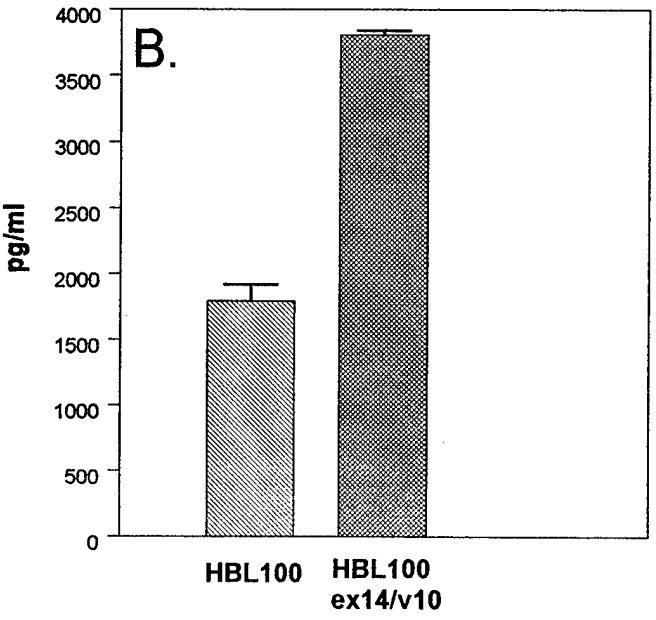


Fig. 4



bFGF



IL-8