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 Y. Bourguignon, Ph.D.

CONTRACTING ORGANIZATION: University of Miami
Miami, Florida 33136

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.

DTIC QUALITY INSPECTED 4

19991015 011

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
	September 1998	Annual (1 Aug 97 - 31 Jul 98)

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 Y. 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)
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)*

CD44 isoforms belong to a family of cell adhesion molecules expressed on the cell surface of many tumor cells during human breast cancer progression. In this study we have analyzed the expression of CD44v3-containing isoforms [containing heparan sulfate addition sites for growth factor binding] in primary breast tumors, axillary nodal metastases and normal breast tissue. Using reverse transcriptase-polymerase chain reaction (RT-PCR) followed by Southern blot, cloning, nucleotide sequencing and RT-in situ-PCR analyses, we have found that at least two CD44v3-containing isoforms, including one new species of CD44v2,Δv3-10 (Δv3 defined as a v3 exon lacking the first 24 base pairs) and another previously reported CD44v3,8-10 are preferentially expressed in human primary breast tumor and axillary nodal metastases but not in normal breast tissues. These finding suggest that these CD44v3-containing isoforms are closely associated with breast cancer metastasis.

Furthermore, we have established a stable transfection of CD44v2,Δv3-10 cDNA into non-metastatic human breast tumor cells (MCF-7) which contain endogenous CD44E isoform. Our results indicate that expression of CD44v2,Δv3-10 in MCF-7 cells promotes tumor cells undergo active cell migration. Treatments of MCF-7 transfectants expressing CD44v2,Δv3-10 with various agents such as anti-$CD44v_3$ antibody, cytochalasin D (a microfilament disrupting agent known to prevent actin polymerization) and W-7 (a calmodulin antagonist) but not colchicine (a microtubule inhibitor), cause a significant inhibition of tumor cell migration. These findings suggest that CD44v2,Δv3-10 (related to human metastatic breast cancers) and associated microfilament components play an important role in the regulation of breast tumor cell migration required for the progression of human breast carcinomas.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	20
	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

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. 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 Bourguignon 8/21/98

PI - Signature Date

TABLE OF CONTENTS

	Page Number
FRONT COVER	1
REPORT DOCUMENTATION PAGE	2
FOREWORD	3
TABLE OF CONTENTS	4
INTRODUCTION	5
BODY	6-9
CONCLUSIONS	10
REFERENCES	11-12
APPENDICES	13-20

(5) INTRODUCTION

The transmembrane glycoprotein CD44 isoforms are all major hyaluronic acid (HA) cell surface receptors that exist on many cell types, including macrophages, lymphocytes, fibroblasts and epithelial cells[1-5]. Due to its widespread occurrence and its role in signal transduction, CD44 isoforms have been implicated in the regulation of cell growth and activation as well as cell-cell and cell-extracellular matrix interactions[1-7].

One of the distinct features of CD44 isoforms is the enormous heterogeneity in the molecular masses of these proteins. It is now known that all CD44 isoforms are encoded by a single gene which contains 19 exons[8]. Out of the 19 exons, 12 exons can be alternatively spliced[8]. Most often, the alternative splicing occurs between exons 5 and 15 leading to an insertion in tandem of one or more variant exons [v1-v10 (exon 6-exon 14) in human cells] 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[9-12]. In particular, CD44v3-containing isoforms have a heparin sulfate addition at the membrane-proximal extracellular domain of the molecule that confers the ability to bind heparin sulfate-binding growth factors[9,10].

Cell surface expression of CD44v isoforms changes profoundly during tumor metastasis, particularly during the progression of various carcinomas including breast carcinomas[13-16]. In fact, CD44v isoform expression has been used as an indicator of metastasis. In this study we have identified at least two CD44v3-containing isoforms, including one new species of CD44v2,Δv3-10 (Δv3 defined as a v3 exon lacking the first 24 base pairs) and another previously reported CD44v3,8-10 which appear to be in a close association with human primary breast tumor and axillary nodal metastases but not in normal breast tissues.

In addition, we have cloned CD44v2,Δv3-10 cDNA into a pRc/CMV vector and transfected this cDNA into non-metastatic human breast tumor cells (MCF-7) containing endogenous CD44E isoform. We have determined that the expression of CD44v2,Δv3-10 in MCF-7 promotes tumor cell migration in a microfilament-dependent manner. These results suggest that the expression of CD44v2,Δv3-10 is involved in breast tumor cell motility required for the progression of human breast carcinomas.

(6) BODY

MATERIALS AND METHODS

Human Breast Carcinoma Samples

Human breast carcinoma samples (e.g. breast carcinomas, axillary nodal metastases and normal breast tissues) were obtained from the tissue bank core facility in the Sylvester Comprehensive Cancer Center at the University of Miami Medical School. These samples were collected after radical mastectomy and characterized pathologically in terms of their stage in malignancy. Specifically, breast tissue samples, 0.5-1.0 cm in diameter, were obtained from fresh surgical resection specimens obtained from patients with breast tumors. The samples were snap-frozen in liquid nitrogen within 10 min of arrival in the pathological specimen reception area and were kept frozen until use. Portions of any lymph node metastasis in the resected tissue have also been collected. Normal breast tissue and lymph node were obtained from the periphery of specimens surgically resected for treatment of cancer, and from other specimens removed for non-neoplastic conditions such as fibrocystic disease of the breast.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and One-Step Cloning

Total RNA was extracted from 200-400 mg of frozen human breast tumor tissue, axillary nodal metastatic tissue, and normal human breast tissue using the acid guanidinium thiocyanate-phenol-chloroform technique of Chomczynski and Sacchi[17]. Approximately 3μg of total RNA was used with an oligo(dT) primer in a reverse transcriptase system (Promega, Madison, WI) to synthesize complementary DNA(cDNA) at 42°C for 1hr., using AMV(avian myeloblastosis virus) reverse transcriptase. After synthesis of the first strand, polymerase chain reaction (PCR) of the CD44cDNA was done by initial melting of the RNA/cDNA at 94°C for 4 minutes, then 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and polymerization at 72°C for 1 minute. The PCR primers were designed initially to look at and amplify the region involved with alternative splicing. Specifically the 2 primers were CD44 exon 4 (exon 4: 5'-TACATCAGTCACAGACCTGC-3') and CD44 exon 14 (exon 14: 5'-CTGCAGTAACTCCAAAGGAC-3'). Analysis of the presence of v3-containing isoforms was facilitated by designing PCR primers within exon 5 and v3 (exon 7). Specifically the primers were CD44 exon 5 (exon 5: 5'-GCACTTCAGGAGGTTACATC-3') and CD44 v3(exon 7) (exon 7: 5'-CTGAGGTGTCTGTCTCTTTC-3'). Analysis for the presence of v2v3-containing isoforms was done by using the previously designed exon 5 left primer and designing a v2v3 specific right junction primer, specifically CD44 v2/v3 (exon 6/7) (exon 6: 5'-GAAGACGTACCAGCCATTTG-3'). The PCR products were one-step cloned using TA-cloning kit (Invitrogen, San Diego, CA) and sequenced by dideoxy sequencing method.

Southern Blot Analysis

The PCR products were separated on a 2% agarose gel, blotted on to the nitrocellulose filter and hybridized to [^{32}P]dCTP-labeled CD44 cDNA probes. In a control experiment, RT-PCR was done in the absence of AMV reverse transcriptase or in the absence of template in the PCR reaction. No amplification products were found by Southern blot in these negative control samples.

Immunohistochemistry and RT *in situ* PCR

The breast tissue specimen was fixed in formalin, embedded in paraffin and sectioned at 4.0 μm thickness. The tissue section was subjected to immunofluorescence staining using rabbit anti-CD44v3 antibody followed by rhodamine-conjugated goat anti-rabbit IgG. For RT *in situ* PCR analysis of CD44v3-containing transcript in breast tissues, a protocol by Nuovo[18] was used with following modifications. In the PCR mixture we used the direct incorporation of fluorescein (FITC)-digoxigenin-11-dUTP and the CD44v3 (exon 7) specific primers (as described above), and 20 PCR cycles were carried out at 50° C for 2 min and 94° C for 1 min. The fluorescent signals were then analyzed by a laser scanning Confocal microscope (MultiProbe 2001 Invert CLSM System, Molecular Dynamics) using a 63 X-oil immersion and an imaging processing device. Images were photographed with Kodak TMAX100 Film.

Cell Lines

The MCF-7 cell line 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 mg/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

MCF-7 cells were transfected with pcDNA3 plasmid (Invitrogen, San Diego, CA) containing the human CD44v2,Δv3-10 cDNA using the LIPOFECTAMINE reagent (Gibco, Grand Island, NY) according to the manufacturer's protocol. Control transfections consisted of either no DNA or pcDNA3 vector DNA. Stable cell lines were established and maintained in Minimum Essential Medium (Gibco) supplemented with 10% FBS, and containing 500 µg/ml G418. 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 µg/ml G418. The growth rate of transfected cells with CD44v2,Δv3-10 appears to be similar to that in the parental cells/vector-transfected control cells.

Analysis of CD44 Expression At the Protein Level

Transfected and parental MCF-7 cells were analyzed by polyacrylamide gel (7.5%) electrophoresis followed by immunobloting with monoclonal rat anti-CD44 antibody (Clone:020; Isotype: IgG_{2b}; obtained from CMB-TECH, Inc., Miami, FL.) which recognizes a common determinant of the CD44 class of glycoproteins, including CD44s and other variant isoforms[14]. The blots were then incubated with peroxidase-conjugated goat anti-mouse IgG (1:10,000 dilution) at room temperature for 1 h. After an addition of peroxidase substrate (Pierce Co.), they were then developed using Renaissance chemiluminescence reagent (Amersham Life Science, England) according to the manufacturers instructions.

Tumor Cell Migration Assays

Twenty-four transwell units were used for monitoring in vitro cell migration as described previously[19]. Specifically, the 8µm porosity polycarbonate filters were used for the cell migration assay[19]. MCF-7 cells [e.g. parental cells (untransfected), control cells (transfected with vector only) or transfectants (containing CD44v2,Δv3-10 cDNA)] [$\approx 1 \times 10^{-4}$ cells/well in phosphate buffered saline (PBS), pH 7.2] [untreated or treated with various agents such as cytochalasin D (20µg/ml), W-7 (20µM), colchicine (1×10^{-5} M), rabbit anti-$CD44v_3$ antibody (50µg/ml) or preimmune serum] were placed in the upper chamber of the transwell unit. The growth medium containing high glucose DMEM supplemented by 10% fetal bovine serum was placed in the lower chamber of the transwell unit. After 18h incubation at 37°C in a humidified 95% air/5% CO_2 atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swap. Cell migration processes were determined by measuring the cells that migrate to the lower side of the polycarbonate filters by standard cell number counting methods as described previously[19]. Each assay was set up in triplicate and repeated at least 3 times. All data were analyzed statistically by Student's t test and statistical significance was set at $p<0.01$.

RESULTS

Identification of CD44 Variant Transcripts Expressed in Human Breast Carcinoma Tissues By RT-PCR, Southern Blot Analyses, cDNA Cloning and Nucleotide Sequencing

In this study we have used the RT-PCR technique to analyze the expression of various CD44 isoforms in both normal and metastatic breast tissues. Our results indicate that normal human breast tissues contain primarily one major band of CD44-related PCR product (Fig. 1, lane 1 and lane 2). This PCR product was subsequently "one-step cloned" into the pCR™ vector from Invitrogen Corporation and sequenced. Our nucleotide sequence data indicate that it represents the CD44 epithelial (CD44E) form (Fig. 1, lane 1 and lane 2). Interestingly, the CD44E form is also expressed in primary breast tumor tissues (Fig. 1, lane 3) and metastatic breast carcinomas (Fig. 1, lane 4). Furthermore, multiple species of the CD44-related gene products [i.e. CD44 variant isoforms (CD44v) have been found in primary breast tumor tissues (Fig. 1, lane 3) and axillary nodal metastases tissues invaded by breast carcinomas cells (Fig. 1, lane 4). These findings are consistent with previous findings showing that several large size CD44 variant isoforms exist in breast carcinoma cells[13-16].

The v3 (or exon 7) insertion of CD44v3 isoforms has been shown to contain heparin sulfate addition sites required for the binding of a wide range of heparin binding growth factors, cytokines, and chemokines for promoting cell growth[9] or angiogenesis[20]. These observations have prompted us to examine the possible expression of CD44v3-containing isoforms in these breast carcinoma tissue specimens. To detect the expression of CD44v3-containing transcript(s) at the RNA level of various breast carcinoma tissues, total RNA from these materials was extracted and analyzed by RT-PCR using exon specific primers. Using a PCR primer pair to amplify between exon 5 and v3 (exon 7) (Fig. 2A) by RT-PCR followed by Southern blot analyses, we have detected the presence of v3-containing species as well as a larger band that represents insertion of the v2 exon along with the v3 exon in the primary breast tumor tissues (Fig. 2, lane 1-6). These two PCR products (v3 and v2v3) were then "one-step cloned" into the pCRII vector from Invitrogen Corp. (San Diego, CA) and sequenced. The nucleotide sequences confirm that they represent v3 and v2v3.

Further analyses indicate that the CD44 isoform containing v2v3 appears to be identical to an amplimer from a human variant CD44 cDNA template containing v2v3 (Fig. 3B, lane 7 and Fig. 3C, lane 8); and is detected only in primary tumors (Fig.3C, lane 1-3) and axillary nodal metastases (Fig. 3C, lane 4-6), and not in normal breast tissues (Fig. 3B, lane 1-5). We believe that the RT-PCR reaction is specific since no amplified fragment can be detected in samples without any reverse transcriptase (Fig. 3B, lane 6; Fig. 3C, lane 7).

Furthermore, PCR products of CD44 amplified between exons 4 and 14 were then analyzed for these v3 and v2v3-containing isoforms. Using the technique of colony hybridization with a v2v3-specific probe, two unique colonies that contained v3 were detected. Using nucleotide sequence analysis, we have found that at least two CD44v3-containing isoforms including CD44v3,8-10 (Fig. 4B) and CD44v2,Δv3-10 (Δv3 defined as a v3 exon lacking the first 24 base pairs) (Fig. 4C) are preferentially expressed in both primary breast tumor tissues (Fig. 1, lane 3; Fig. 3, lane 1-3) and axillary nodal metastases (Fig. 1, lane 4; Fig. 3, lane 4-6) but not in normal breast tissues (Fig. 1, lane 1 and 2; Fig. 3, lane 1-5).

Immunoflurescence and RT *in situ* PCR

The expression of CD44v3-containing transcripts and proteins were also analyzed in breast carcinoma tissues by RT *in situ* PCR analysis (Fig. 5A) and immunoflurescence staining (Fig. 5B), respectively. The results of the RT *in situ* PCR using the v3 (exon 7) specific primer pair show the presence of strong fluorescent signal in the breast carcinomas tissues (Fig. 5A). This signal is absent in the negative control samples where the reverse transcriptase was eliminated during the RT reaction (data not shown). The location of CD44v3-containing proteins in breast carcinomas tissues is also established by immunofluorescence staining using anti-CD44v3 antibody (Fig. 5B). The fact that

both CD44v3-containing RNA transcripts and proteins are colocalized in the same population of breast carcinomas (Fig. 5C) suggests that the CD44v3-containing transcripts (e.g. CD44v2,Δv3-10 and CD44v3,8-10) are successfully expressed at the protein level in breast carcinoma tissues.

Transfection and Expression of CD44v2,Δv3-10 In Human Breast Tumor Cells (MCF-7) Containing CD44

In this study certain CD44 variant (CD44v) isoforms, such as CD44v3,8-10 and CD44v2,Δv3-10 (Fig. 2 and 3), have been found to be preferentially associated with human breast carcinomas cells in both early primary tumors and the advanced stages of axillary nodal metastases (Fig. 3). The biochemical characteristics of the CD44v3,8-10 isoform (but not the CD44v2,Δv3-10 isoform) has been described previously by a number of investigators[9,20]. Consequently, we have decided to focus on analyzing the structural and functional properties of this new species of CD44v3-containing isoforms such as CD44v2,Δv3-10 isoform. In order to test directly whether the expression of CD44v2,Δv3-10 isoform is involved in breast tumor cell migration, we have established a new, stable transfectant of a breast tumor cell line which is capable of expressing CD44v2,Δv3-10 on its cell surface. Specifically, CD44v2,Δv3-10 cDNA was cloned into a pRc/CMV vector and expressed in non-metastatic human breast tumor cells (MCF-7) containing endogeneous CD44E (but not CD44v2,Δv3-10). Using anti-CD44-mediated immunobloting techniques, we have found that both the parental (untransfected) cells and control cells (transfected with vector only) contain only one large endogeneous CD44E isoform (M.W. ≈ 160kDa) on the cell surface (Fig. 6A and 6B). In cells transfected with CD44v2,Δv3-10 cDNA, one additional CD44-related polypeptide (M.W. ≈280kDa) plus CD44E is expressed on the cell surface (Fig. 6C). Routinely, we selected at least 8 cloned MCF-7 transfectants from each transfection experiment based on their ability to express CD44v2,Δv3-10 (≈280kDa) on the cell surface. Since all 8 cloned transfectants express a very similar level of CD44v2,Δv3-10 (≈280kDa), in this study we have chosen one representative MCF-7 transfectant [expressing CD44v2,Δv3-10 (≈280kDa) (Fig. 6C)] to address the effects of CD44v2,Δv3-10 expression on tumor cell motility as described below.

Analysis of Tumor Cell Migration of MCF-7 Transfectants Expressing CD44v2,Δv3-10

One of the common properties of all metastatic tumor cells is their ability to undergo cell movement and migration. In this study using in vitro migration assays, we have found that the MCF-7 transfectants expressing CD44v2,Δv3-10 display a very active migration capability (Table 1). On the contrary, both the parental (untransfected) cells and control cells (transfected with vector only) display relatively low migration activities (Table 1). Treatments of the MCF-7 transfectant (containing CD44v2,Δv3-10 cDNA) with various agents, such as anti-$CD44v_3$ antibody, cytochalasin D (a microfilament disrupting agent known to prevent actin polymerization) and W-7 (a calmodulin antagonist) but not colchicine (a microtubule inhibitor) (Table 1), cause a significant inhibition of tumor cell migration (Table 1). These findings suggest that CD44v2,Δv3-10 and associated microfilament components play an important role in the regulation of breast tumor cell motility.

(7) CONCLUSIONS

CD44 variant isoforms have been shown to be closely involved in the onset of tumor development and metastasis[13-16]. Moreover, certain CD44 variant (CD44v) isoforms appear to be expressed at high levels on the surface of tumor cells during tumorigenesis and metastasis[13-16]. For example, one of the CD44v isoforms, CD44v6 has been correlated with an advanced tumor stage and poor patient survival in non-Hodgkin's lymphoma[21] and colorectal tumors[22]. This CD44v6 isoform has also been shown to confer metastatic behavior on rat pancreatic cells in a spontaneous metastasis assay[23]. In addition, CD44v5 expression has been proposed as an early tumor marker for colorectal carcinoma since it is detectable on dysplastic colon polyps and carcinoma, yet is not present on normal intestinal epithelium[22]. Furthermore, increased CD44v7,8 expression has been found during the progression of human cervical carcinoma, showing its presence in almost 100% of tissue samples at the stage of carcinoma in-situ[24]. A positive association between the presence of CD44v9 and the progression of human gastric carcinoma has also been demonstrated[25]. Recently, CD44v10 has also been shown to be closely associated with breast tumor development and progression[14,28].

As the histologic grade of each of the tumors progresses, the percentage of lesions expressing an associated CD44 variant isoform increases. In particular, the CD44v3-containing isoforms are expressed preferentially on highly malignant breast carcinoma tissue samples. In fact, there is a direct correlation between CD44v3 isoform expression and increased histologic grade of the malignancy[14,26]. One study indicates that breast tumor expression of the CD44v3 isoform may be used as an accurate predictor of overall survival (e.g. nodal status, tumor size, and grade)[14,27]. In this study we have used the techniques of RT-PCR and DNA sequence analysis to study the expression of CD44 variant isoforms in primary breast tumors, axillary nodal metastases and normal breast tissue. Our data indicate that both primary breast tumor tissues (Fig. 1, lane 3) and metastatic breast carcinomas (Fig. 1, lane 4) reveal a high level of CD44 variant isoform expression compared to the very low level seen in normal breast tissue (Fig. 1, lane 1 and lane 2). These results are consistent with previous findings linking the expression of various CD44 variant isoforms to tumor progression and metastasis in several types of cancers[14,21-28].

When analyzed for the presence of the v3 exon, we have found that primary tumors and axillary metastases contain v3 as well as v2v3 while normal breast tissue does not (Fig. 2 and Fig. 3). We have identified two CD44 variant isoforms (CD44v2,Δv3-10 and CD44v3,8-10) (Fig. 4) that contain v3 and are present in breast tumors and metastases, but not in normal breast tissue. The fact that both CD44v3 RNA transcripts and proteins are localized (Fig. 5) in the same population of breast carcinoma cells by RT-in situ-PCR and immunofluorescence staining suggests that these CD44v3-containing isoforms are closely associated with the breast carcinoma progression.

The metastatic phenotype of tumor cells is characterized by tumor cell motility[29,30] and is clearly linked to cytoskeletal function. Dissection of the transmembrane pathways controlling these cellular processes should aid in understanding the regulatory mechanisms underlying tumor metastasis. Previously, we have demonstrated that certain CD44 isoforms [e.g. CD44s and CD44 variant (ex14/v10)] display high affinity binding to the cytoskeletal protein, ankyrin[6,31-37]. In this study we have found that MCF-7 transfectants expressing CD44v2,Δv3-10 undergo active cell migration processes as compared to parental cells (untransfected) or control cells (vector-transfected cells) (Table 1). These data suggest that CD44v2,Δv3-10 may be required for breast tumor cell migration. Treatment of these MCF-7 transfectants expressing CD44v2,Δv3-10 with certain agents, including anti-CD44v3, cytochalasin D (a microfilament inhibitor) and W-7 (a calmodulin antagonist) but not colchicine (a microtubule disrupting agent), effectively inhibit tumor cell migration (Table 1). Therefore, we believe that this human breast tumor-specific isoform (CD44v2,Δv3-10) plays an important role in promoting the cytoskeleton function leading to tumor cell migration which is required for human breast cancer progression. Future work using a larger number of metastatic breast cancer samples is needed in order to further establish these newly described CD44 variant isoforms as potential markers for monitoring the progression of human breast cancer metastasis.

(8) REFERENCES

1. Lesley J, Hyman R, Kincade, PW. CD44 and its interaction with extracellular matrix. Adv. Immunol 1993;54:271-335.
2. Picker LJ, Nakache M, Butcher EC. Monoclonal antibodies to human lymphocyte homing receptors define a novel class of adhesion molecules on diverse cell type. J Cell Biol 1989;109:927-937.
3. Bourguignon LYW, Lokeshwar VB, He J, Chen X, Bourguignon GJ. A CD44-Like Endothelial Cell Transmembrane Glycoprotein (GP116) Interacts With Extracellular Matrix and Ankyrin. Mol Cell Biol 1992;12:4464-4471.
4. Zhu D, Bourguignon LYW. Overexpression of CD44 in p185^{neu}-transfected NIH3T3 cells promotes an up-regulation of hyaluronic acid-mediated membrane-cytoskeleton interaction and cell adhesion. Oncogene 1996; 12:2309-2314.
5. Brown TA, Bouchard T, St. John T, Wayner E, Carter WG. Human keratinocytes express a new CD44 core protein (CD44E) as a heparin-sulfate intrinsic membrane proteoglycan with additional exons. J Cell Biol 1991; 113:207-221.
6. Bourguignon LYW. Interaction between membrane-cytoskeleton and CD44 during lymphocyte signal transduction and cell adhesion. Current Topics in Membranes. (W.J. Nelson, ed.) 1996;43:293- 312.
7. Stamenkovic I, Amiot M, Pesando, JM, Seed B. The hemopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyalurononan-bearing cells. EMBO J 1991;10:343-347.
8. Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI. Genomic structure of DNA coding the lymphocyte homing receptor CD44 reveals at least 12 alternative spliced exons. Proc. Natl. Acad. Sci. (USA) 1992; 89:12160-12164.
9. Bennett, K, Jackson, D, Simon, J, Tanczos, E, Peach, R, Modrell, B, Stamenkivic, I, et al., CD44 isoforms containing exon v3 are responsible for the presentation of heparin-binding growth factor, Journal of Cell Biology 1995;128: 687-698.
10. Jackson DG, Bell JI, Dickinson R, Timans J, Shields J, Whittle N. Proteoglycan forms of the lymphocyte homing receptor CD44 are alternatively spliced variants containing the v3 exon. J Cell Biol 1995;128:673-685.
11. Jalkanen SP, Jalkanen M. Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. J Cell Biol 1992; 116:817-825.
12. Lokeshwar VB, Bourguignon LYW. Post-translational protein modification and expression of ankyrin-binding site(s) in GP85 and its biosynthetic precursors during T-lymphoma membrane biosynthesis. J. Biol. Chem. 1991; 266:17983-17989.
13. Dall, P., Heider, K.-H., Sinn, H.-P., Skroch-Angel, P., Adolf, G., Kaufmann M, Herrlich P, Ponta H. Comparison of immunohistochemistry and RT-PCR for detection of CD44v-expression, a new prognostic factor in human breast cancer. Int. J. Cancer 1995;60:471-477.
14. Iida N, Bourguignon LYW. New CD44 splice variants associated with human breast cancers. J Cell Physiol 1995;162:127-133.
15. Kaufmann M, Meider KH, Sinn HP, von Minckwitz G, Ponta H, Herrlich P. CD44 variant exon epitopes in primary breast cancer and length of survival. Lancet 1995;345:615-619.
16. Rodriguez C, Monges G, Rouanet P, Dutrillaux B, Lefrancois, D, Theillet C. CD44 expression patterns in breast and colon tumors: A PCR-based study of splice variants. Int. J. Cancer 1995; 64:347-354.
17. Chomczynski P, Sacci N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phonol chloroform extraction. Anal. Biochem 1987;162:156-159.
18. Nuovo GJ. RT in situ PCR with direct incorporation of digoxigenin-11-dUTP: Protocol and applications. Biochemica 1994;11: 4-19.
19. Merzak A, Koochekpour S, Pilkington GJ. CD44 mediates human glioma cell adhesion and invasion in vitro. Cancer Res. 1994;54:3988-3992.
20. Bourguignon LYW, Gunja-Smith Z, Iida, N, Zhu, HB, Young LJT, Cardiff RD. CD44v3 is responsible for vascular growth factor (VEFG) binding, membrnae-cytoskeleton interaction, and matrix matalloproteinase (MMP) activation in metastatic breast tumor cells. Pro Amer Ass Can Res 1997; 38:532.

21. Koopman G, Heider KH, Horst E, Adolf GR, van den Berg F, Ponta, H, Herrlich, P, et al., Activated human lymphocytes and aggressive non-Hodgkin's lymphomas express a homologue of the rat metastasis-associated variant of CD44. J Exp Med 1993;177:897-904.
22. Herrlich, P, Pals, S, Ponta, H, CD44 in colon cancer. Eur J. Cancer 1995;31A:1110-1112.
23. Gunthert U, Hofmann M, Rudy M, Reber S, Zoller M, Haussmann I, Matzku, S, et al., A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell 1991;65:13-24.
24. Dall P, Hekelf A, Ikenberg H, Goppinger A, Bauknecht T, Pfleiderer A, Moll J, et al. Increasing incidence of CD44v7/8 epitope expression during uterine cervical carcinogenesis, Int. J. Cancer 1996;69:79-85.
25. Mayer B, Jauch KW, Gunthert U, Figdor CG, Schildberg F, Funke I, Johnson J, De-novo expression of CD44 and survival in gastric cancer. Lancet 1993;342:1019-1022.
26. Sinn HP, Heider KH, Skroch-Angel P, von Minckwitz G, Kaufmann M, Herrlich P, Ponta H, Human mammary carcinomas express homologues of rat metastasis-associated variants of CD44 Breast Cancer Research and Treatment 1995;36:307-313.
27. Kaufmann M, Heider KH, Sinn HP, von Minckwitz G, Ponta H, Herrlich P, CD44 variant exon epitopes in primary breast cancer and length of survival, Lancet 1995;345:615-619.
28. Iida N, Bourguignon, LYW, Coexpression of CD44 variant (v10/ex14) and CD44s in human mammary epithelial cells promotes tumorigenesis, J Cell Physiology 1997;171:152-160.
29. Jiang WG, Puntis MCA, Hallett MB. Molecular and cellular basis of cancer invasion and metastasis: implications for treatment. British J. Surgery 1994; 81:1576-1590.
30. Lauffenburger DA, Horwitz AF. Cell migration: A physically integrated molecular process. Cell 1996; 84:359-369.
31. Bourguignon LYW, Kalomiris, EL, Lokeshwar VB. Acylation of the Lymphoma Transmembrane Glycoprotein, GP85, May be Required for GP85-Ankyrin Interaction. J Biol Chem 1991; 266:11761-11765.
32. Kalomiris EL, Bourguignon, LYW. Lymphoma Protein Kinase C Is Associated with The Transmembrane Glycoprotein, GP85, and May Function In GP85-Ankyrin Binding. J Biol Chem 1989;264:8113-8119. 33. Bourguignon LYW, Lokeshwar VB, Chen X, Kerrick, WGL. Hyaluronic Acid (HA)-Induced Lymphocyte Signal Transduction and HA Receptor (GP85/CD44)-Cytoskeleton Interaction. J Immunol 1993; 151: 6634-6644.
34. Bourguignon LYW, Iida N, Welsh, CF, Krongrad, A, Zhu, D, Pasquale D. Involvement of CD44 and Its Variant Isoforms In Membrane Cytoskeleton Interaction, Cell Adhesion and Tumor Metastasis. J. Neuro-Oncology 1995; 26:201-208.
35. Lokeshwar VB, Fregien N, Bourguignon, LYW. Ankyrin binding domain of CD44 (GP85) is required for the expression of hyaluronic acid-mediated adhesion function. J Cell Biol 1994; 126:1099-1109. 36. Lokeshwar VB, Bourguignon, LYW. The Lymphoma Transmembrane Glycoprotein GP85 (CD44) Is A Novel G-Protein Which Regulates GP85 (CD44)-Ankyrin Interaction. J Biol Chem 1992; 267:22073-22078.
37. Lokeshwar VB, Iida N, Bourguignon, LYW. The cell adhesion molecule, GP116 is a new CD44 variant (ex14/v10) involved in hyaluronic acid binding and endothelial cell proliferation. J. Biol. Chem. 1996; 271:23853-23864.

(9) APPENDICES

Table 1: Effects of Various Agents On Tumor Cell Migration.

Treatments[a]	Cell Migration[b] (Cell Number/Field)[c]
Parental Cells (untransfected)	10
Control Cells (transfected with vector only)	15
Transfectants (containing CD44v2,Δv3-10 cDNA)	155
Transfectants treated with anti-CD44v_3 antibody	54
Transfectants treated with preimmune serum	149
Transfectants treated with cytochalasin D	36
Transfectants treated with W-7	24
Transfectants treated with Colchicine	152

a: The concentrations of various reagents used in this experiment were: rabbit anti-CD44v_3 (50µg/ml); cytochalasin D (20 µg/ml); W-7 (20 µM); colchicine (1 x 10^{-5} M).
b: The procedures for the in vitro migration assay were described in the Materials and Methods.
c: The values expressed in this table represent an average of triplicate determinations of 3-5 experiments with a standard deviation less than ±5%.

Fig.1: A representative RT/PCR and Southern blot analyses of CD44 isoforms using RNAs isolated from human breast tissues.

Total RNA isolated from breast tissues were reverse-transcribed and subjected to PCR primer pairs (exon 4 and exon 14) as described in Fig. 1A. Subsequently, RT-PCR products were analyzed by Southern blot hybridization as shown in Fig. 1B (lane 1 and 2: normal breast tissues; lane 3: primary breast tumor tissues; lane 4: axillary nodal metastatic breast tumor tissues). [CD44E, CD44 epithelial form; CD44v, CD44 variant isoforms].

Fig. 2: RT/PCR and Southern blot analyses of v3-containing CD44 isoforms using RNAs isolated from human primary breast tumor tissues.

Total RNA isolated from primary breast tumor tissues were reverse-transcribed and subjected to PCR using PCR primer pairs [exon 5 and v3 (exon 7)] as described in Fig. 2A. Subsequently, RT-PCR products were analyzed by Southern blot hybridization as described in the Materials amd Methods. B: Southern blot reveals the presence of v3-containing CD44 transcripts (V3) as well as a larger band that represents insertion of the v2v3 exon as well in the primary breast tumor tissues (lane 1-6). These two PCR products (v3 and v2v3) were then cloned into the pCRII vector (Invitrogen Corp., San Diego, CA) and sequenced as described in the Materials and Methods. The nucleotide sequences confirm that these species belong to v3 and v2v3.

Fig. 3: RT/PCR and Southern blot analyses of v2v3-containing CD44 isoforms using RNAs isolated from human breast tissues.

Total RNA isolated from breast tissues were reverse-transcribed and subjected to PCR using PCR primer pairs [exon 5 and the V2V3 Junction] as described in Fig. 3A. Subsequently, RT-PCR products were analyzed by Southern blot hybridization as shown in Fig. 3B (lane 1-5: normal breast tissues) and Fig. 3C (lane 1-3: primary breast tumor tissues; lane 4-6: axillary nodal metastatic breast tumor tissues). As a negative control, RT-PCR was carried out in the absence of reverse transcriptase [B (normal breast tissues):lane 6; C (metastatic breast tumor tissues:lane 7)]. As a positive control, a known CD44 variant isoform which contains v2v3 exon insertions was used [B (normal breast tissues):lane 7; C (metastatic breast tumor tissues:lane 8].

Fig. 4: Exon map of CD44 in human (A) and schematic illustration of the $CD44v_{3,8-10}$ isoform (B) and CD44v2,Δv3-10 isoform (C) detected in primary breast tumor tissues and axillary lymph nodal metastases as described in Figs. 1-3.

Fig. 5: RT *in situ* PCR analysis of v3-containing CD44 transcripts and proteins in breast carcinomas tissues.

Breast carcinomas tissue specimen was fixed with formalin, paraffin embedded, sectioned (4 μm) and processed for RT *in situ* PCR and immunofluorescence staining as described in the Materials and Methods.

A: Detection of CD44v3-specific transcripts by RT *in situ* PCR using an v3-specific primer pair and FITC-digoxigenin-11-dUTP as described in the Materials and Methods.

B: Immunofluorescence staining of CD44v3-containing proteins using rabbit anti-CD44v3 antibody followed by rhodamine-conjugated goat anti-rabbit IgG.

C: Colocalization of Cd44v3-specific transcripts (A) and proteins (B) by combining two images (A and B) together.

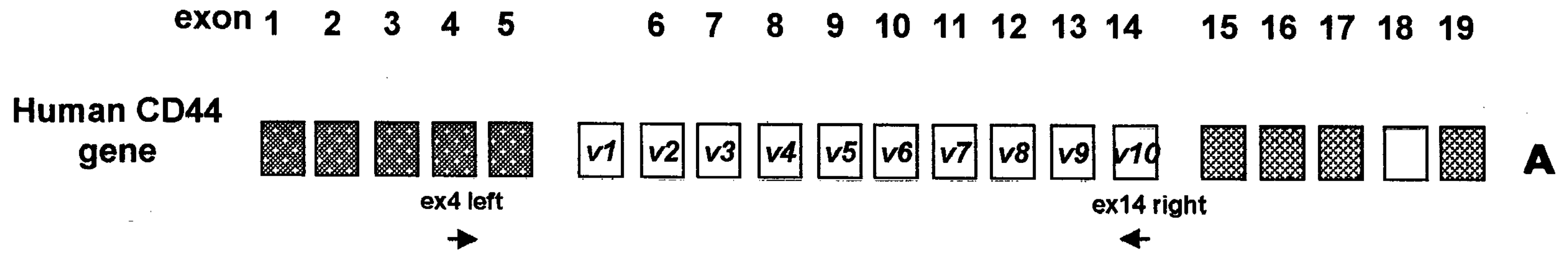
Fig. 6: Analysis of CD44v2,Δv3-10 isoform expression in MCF-7 transfectants by monoclonal rat anti-CD44-mediated immunoblot.

Lane A: Anti-CD44-mediated immunoblot of the cell lysate obtained from parental (untransfected) MCF-7 cells [revealing the presence of CD44E (≈160 kDa)].

Lane B: Anti-CD44-mediated immunoblot of the cell lysate obtained from the control (vector-transfected) MCF-7 cells [revealing the presence of CD44E (≈160 kDa)].

Lane C: Anti-CD44-mediated immunoblot of the cell lysate obtained from MCF-7 transfectants containing CD44v2,Δv3-10 cDNA [revealing the presence of CD44v2,Δv3-10 (≈280 kDa) plus CD44E (≈160 kDa)].

Fig. 1



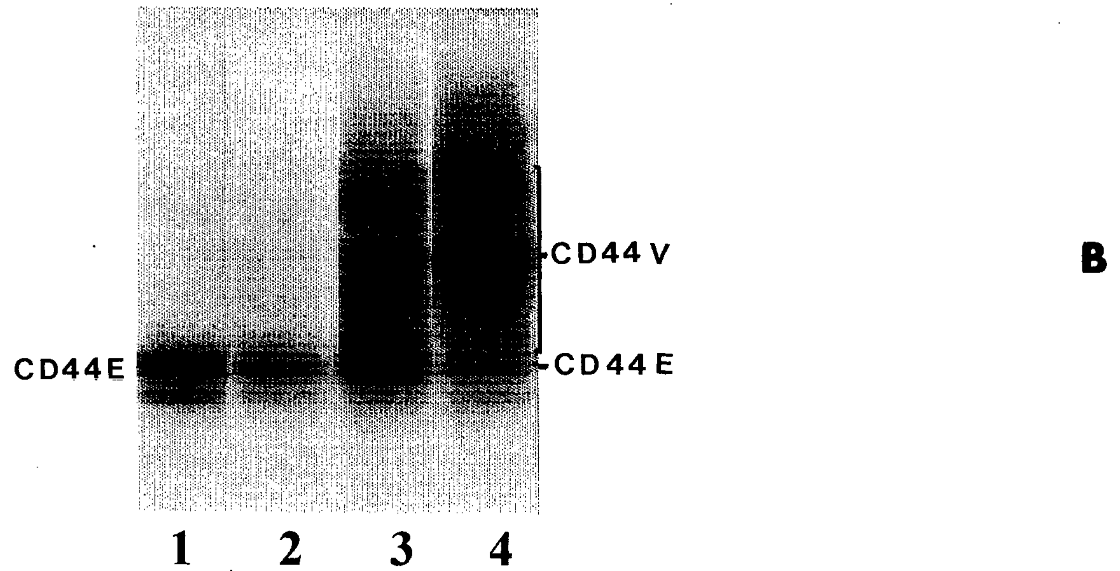


Fig. 2

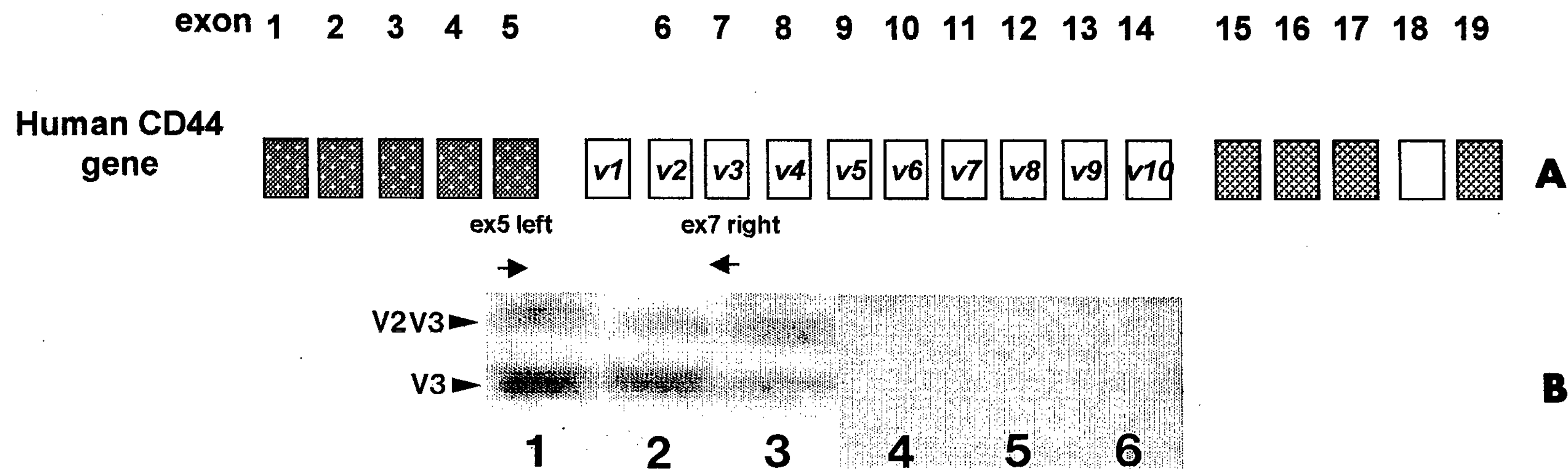


Fig. 3

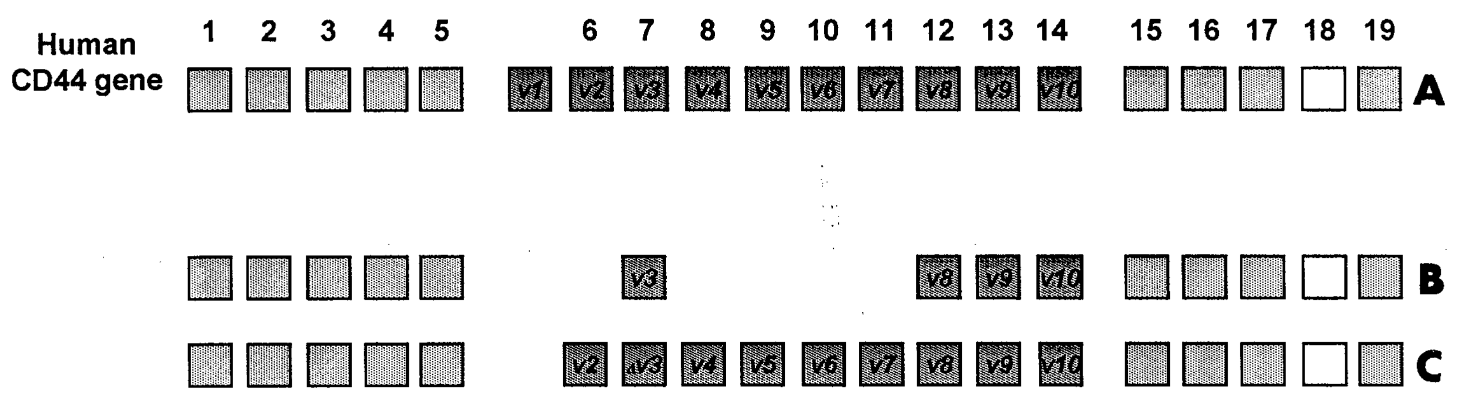


Fig. 4

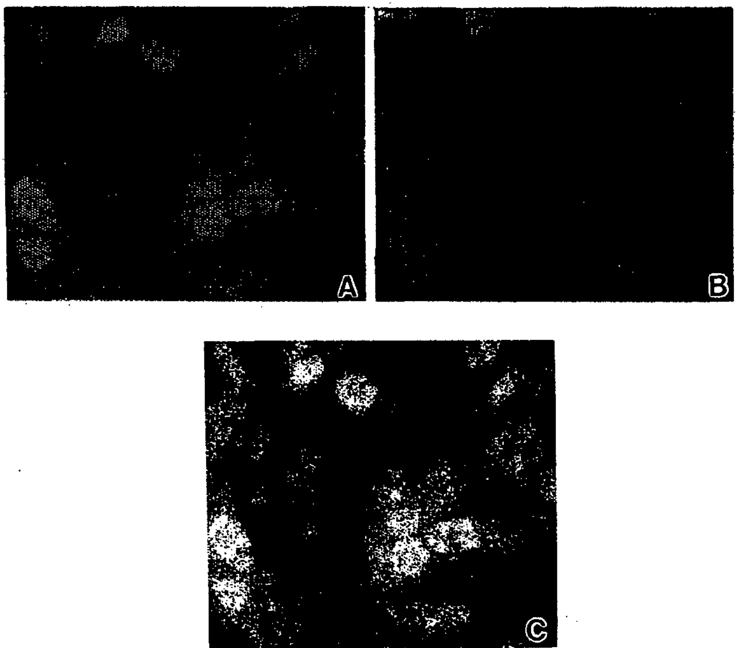


Fig. 5

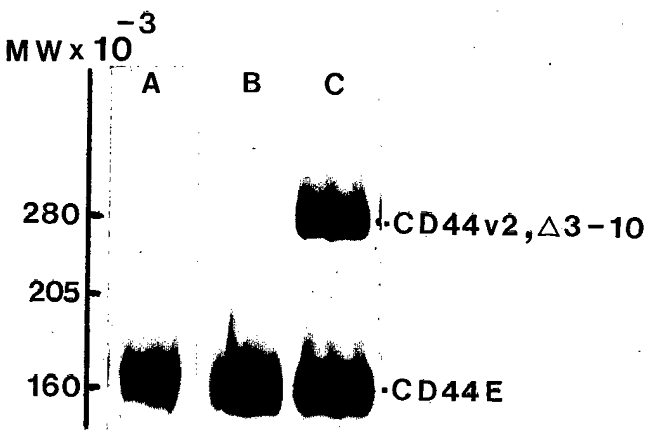


Fig. 6