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13. ABSTRACT (Maximum 200) The deadly consequences of breast cancer are due to metastasis, a process in which tumor cells penetrate the blood vessels and enter other tissues to spread the cancer. This movement through vessels and tissues is attributed to a group of digestive enzymes (the matrix metalloproteinases or MMPs) that can destroy the matrix in advance of tumor cell movement. These MMPs are normally produced in small amount and are held in check by inhibitors in the tissues (tissue inhibitors of MMPs or TIMPs). We took 125 samples of breast tissues (benign tumors and various carcinomas) and measured the production of six different MMPs and 2 TIMPs in a unified multipronged approach. We used antibody methods to see which cells are producing these enzymes and inhibitors. The most prominent enzyme was MMP-9, also known as gelatinase B, which is able to break down the wall that forms around tumor cell clusters. While this, and other MMPs were elevated in cancer, the TIMP inhibitors were produced at levels well below normal. This results in an imbalance in which the destructive proteases greatly outweigh the controlling inhibitors, facilitating the spread of the cancer. Biochemical quantification of MMPs by zymography showed an overall increase in all types of MMPs in cancer tissues. MMP-9 was the key MMP; it was present at levels 0.35 (<i>in situ</i> ductal) to 3.5 (infiltrating ductal) µg/g wet weight tissue in cancer tissues compared to unquantifiable amounts in normal (3/6) and benign neoplasm (16/20) tissues. Zymography also showed a fraction of MMP-9 and MMP-2 in their active forms in grade III breast cancers compared to normal and benign tissues. Reverse zymography showed the presence of TIMPs -1, -2 and -3 in all breast tissues. Quantitation by TIMP-1 and TIMP-2 ELISA kit clearly showed that total amounts of TIMPs were lower in cancer (1.49 µg/g wet weight tissue in adenocarcinomas compared to higher amounts in normal (4.5 µg) and benign (5.6µg) tissues. The production of TIMPs in cancer tissues falls far short of the amount needed to counteract the excessive production of MMPs leading to an imbalance in enzyme and inhibitor expression. Quantitation of MMP and TIMP gene expression (MMP-9, -2, -1, TIMP-1 and -2) by RT-PCR method. are in agreement with both zymography and immunohistochemical findings.				
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FOREWORD

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Zeevat Gupta-Smith 9/30/98
PI Signature Date

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ROLE OF MATRIX METALLOPROTEINASES AND THEIR TISSUE INHIBITORS IN HUMAN BREAST ADENOCARCINOMA**(5) INTRODUCTION****BACKGROUND**

The overall objective of this research is to explore the role of matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) in the invasive and metastatic processes of human breast tumors [1-4]. The hypothesis to be tested is that an imbalance may arise through normal or elevated production of MMPs accompanied by a diminished production of inhibitory TIMPs. Metastasis of an initially localized tumor to vital organs is the dominant cause of cancer related deaths [5]. The mechanisms controlling the metastatic progression of a localized tumor are very complex, involving many biochemical and cellular events [6,7]. Recent evidence indicates that secreted matrix metalloproteinases (MMPs) not only play a major role in the penetration of the basement membrane surrounding the tumor [8] but also play a role in the growth of primary and secondary tumors [9].

The most important traits of tumor cells are their ability to specifically attach to the extracellular matrix (ECM), degrade this extracellular barrier so as to escape out of the primary location into the circulation and establish themselves at the site of metastasis. The breakdown of the ECM can be traced to the action of one or more members of the family of zinc proteases named **matrix metalloproteinases (MMPs)** secreted as proenzymes and are activated outside the by the removal of the 9 kDa prosegment from the active site by proteases [10]. Three enzymes are most likely responsible for the degradation of ECM [11]: 72 kDa gelatinase (gelatinase A, type IV collagenase, MMP-2), 92 kDa gelatinase (gelatinase B, type V collagenase, MMP-9), and the 57 kDa stromelysin (MMP-3) [12-14]. 72 kDa gelatinase was implicated in human breast cancer where 36/40 cases of invasive tumors were positive by immunohistochemistry [15]. Stromelysin-3 and an interstitial collagenase (MMP-1) also have been implicated in human breast cancer [16,17,18].

Metalloproteinase action is limited by specific inhibitors, TIMPs. These are small proteins produced by cells (fibroblasts and tumor cells) and neutralize the destructive activities of MMPs and play an important role in controlling ECM degradation. There are four species that may be implicated in tumors: TIMP-1, TIMP-2, TIMP-3 and TIMP-4 of relative mass 28 kDa, 20 kDa, and 23 kDa and 22 kDa respectively [10,19,20]. They bind to activated MMPs in a 1:1 molar ratio and inhibit their activity. TIMP-2 and TIMP-3 share an amino acid homology with TIMP-1, although they are encoded by different genes [20]. Moreover, many cells produce MMP-2 in a 1:1 complex with TIMP-2 and TIMP-4; similarly, MMP-9 usually comes with an associated molecule of TIMP-1. The TIMP molecule in these cases is bound to the C-domain of the enzyme, but interferes with the activation of the latent gelatinases (MMP-2 and MMP-9).

Since normal cells as well as non-invasive cells produce MMPs and also TIMPs, it seems likely that the extent of activation of MMPs and the levels of inhibitory TIMPs may be two key factors in the progression of normal to invasive cell type. Elevated levels of MMP-9

have been detected in the plasma of patients with breast cancer [21] although no such correlation was found for MMP-2. Not all of the MMPs and TIMPs need be related to the tumor cells. It is quite likely that tumor cells can stimulate neighboring stromal cells through cytokines to produce enzymes that degrade the matrix. So in certain breast tumors and other cancers of skin and colon, 92 kDa gelatinase was found at the tumor/stroma border but not in tumor cells [22].

In summary, there is a compelling evidence of a role for metallo-proteases and the TIMP inhibitors in tumor cell invasiveness. There are lines of evidence to support the concept that an increase in gelatinases and a decrease in TIMPs may be important in this process together with a greater conversion of latent to active forms of the MMPs. The resultant increased MMP activity would permit penetration of basement membrane surrounding the tumor by cells which produce these enzymes or stimulate neighboring stromal cells to produce such enzymes. The same processes would also be important in metastasis of the tumor cells

The underlying hypothesis to be tested is that the invasiveness of human breast cancer is dependent on the action of specific metalloproteinases that can degrade the constraining basement membranes of the extracellular matrix. This action, in turn, depends on two factors - how much active form of enzyme is present and how much inhibitory TIMP is present. Our hypothesis is that an imbalance occurs such that invasive cells produce (or cause to be produced) more active enzyme and less inhibitor than normal cells or cells of benign tumors.

In order to test the hypothesis, the following types of studies were planned. i) Identification and localization of various MMPs and TIMPs by immunohistochemistry by the use of specific anti-MMPs and anti-TIMPs IgGs. ii) Determination of the amounts and ratios of active and latent enzyme by substrate zymography and immuno-precipitation combined with zymography. iii) Determination of mRNA levels for the MMPs and TIMPs in the same tissue samples, based on Northern blotting or RT-PCR analysis. iv) Culture of epithelial cells and fibroblastic cells growing out from explants of human mammary carcinomas and determination of their production of active and latent MMPs and TIMPs.

(6). BODY

6.A EXPERIMENTAL METHODS

6 A.1. Tissue Samples: The tissues (~ 1 cm³, snap frozen, stored at -70° C) diagnosed as displaying breast carcinoma, normal or benign are obtained from the Tissue Procurement laboratory, Pathology Department, University of Miami Medical School, Miami Florida. The Pathology report for each tissue used is well studied by Dr. Scott Sittler, Associate Professor, Department of Pathology and identified as a pathologist for this project. Corresponding to each frozen sample there are many paraffin-fixed blocks, and with Dr. Sittler's help, care is taken to screen various paraffin-fixed blocks to obtain a block of adjacent tissue to be used for immunohistochemistry.

The Florida tumor registry can provide the history of patients showing the development of metastasis, survival rate of the patients up to five years and beyond. This service will help in collating the results in the next six months. The snap frozen tissues kept for five years are suitable in some instances for Northern (mRNA estimation) and most of them for RT - PCR as long as care is taken during the preparation of total tissue RNA

Informed consent forms are not needed for this study. In accordance with 21 CFR 56.111(a)(3), expedited review (#97/480) has been approved by the University of Miami's Human Subjects Institutional Review Board for use of residual tissues for the year 1998-1999.

6.A.2. Generation of antibodies (IgGs) and Western blotting: Monospecific antibodies were raised in rabbits against the whole molecule of enzyme stromelysin (MMP-3 from human cartilage), TIMP-1 from human cartilage chondrocytes, and TIMP-2 from skin fibroblasts. Pro-segment peptides for 92 kDa, 72 kDa, and stromelysin (MMP-1), metal binding region peptides of 92 kDa and 72 kDa and N-terminal peptides of active enzyme, MMP-1 (interstitial collagenase). Purified peptides (15-17 amino acids, prepared by the Peptide Laboratories of Department of Biochemistry and Molecular Biology using Advanced Chemtech automatic synthesizer model ACT350) were conjugated to bovine serum albumin, ovalbumin or hemocyanin, dialyzed, and injected into rabbits to raise the antibodies that were characterized for their reactivity to other MMPs using Western blot analysis [23].

6.A.3. Immunohistochemistry: These analyses are carried out in Principal Investigator's laboratory using Histostain™ SP Kit (Zymed Laboratories, San Francisco, CA) according to manufacturer's instructions with minor modifications. The stained slides are reviewed by Dr. Scott Y. Sittler, Department of Pathology. Paraffin sections (4 microns) are cleared of paraffin, blocked for endogenous peroxidase, washed in water, PBS, blocked with normal horse serum and then are treated with drops of specific primary antibodies in a humidity chamber (1h). The tissues are washed, and treated with a biotinylated secondary antibodies (1-3h), followed by avidin-biotin-peroxidase complex [24]. They are then washed and treated with the chromogen. The slides are counterstained with hematoxylin, washed, dehydrated and evaluated for the localization of various MMPs and TIMPs in specific cells in the tissue. The kit incorporates HorseRadish Peroxidase (HRP), streptavidin, and affinity-purified antibodies into the Labeled-

[strept]Avidin-Biotin (LAB-SA) method. The chromogen/substrate system [aminoethyl carbazole (AEC) for a red signal or diaminobenzidine (DAB) for a brown signal] creates an intense color deposit around the antigen/antibody/enzyme complex in the tissue or cell sample.

6.A.4.Extraction and assays of metalloproteinases and inhibitors:

Human breast tissues are weighed (100-200 mg), minced (finely) and homogenized (in the hood) in 7.5 volumes extraction buffer (0.25% Triton-X 100 or 1 M GuHCL or 0.5 - 2% SDS in 50 mM Tris/HCl buffer, pH 7.5) using Polytron homogenizer, centrifuged and the pellet reextracted with 2.5 volumes of appropriate extraction buffer, centrifuged and supernatants combined [25]. All steps are carried out at about 4°C. Extracts (except SDS) are dialyzed and stored in aliquots at -70°C.

Most tissues contain inhibitory activity which appears to be TIMP (tissue inhibitor of metalloproteinases). These can be destroyed, without affecting the metalloproteinases, by reduction (2 mM DTT) and alkylation [26]. This step also destroys any alpha-2-macroglobulin. Protein estimation of homogenates or concentrated media are by the use of BioRad protein estimation kit.

Tissue extracts (with or without reduction and alkylation) and column fractions (molecular seive or affinity chromatograophy) are assayed and quantitated for various MMPs using (1) ³H-acetylated Type I gelatin to estimate the MMP-2 and MMP-9 gelatinases [25] (2) ³H-acetylated Type I rat skin collagen for MMP-1 (interstitial collagenase [25] and ³H-carboxymethylated transferrin [27] ³H-acetylated proteoglycan monomer bead assay [23] for stromelysin (MMP-3) and MMP-7. Blanks are set up with 1,10-phenanthroline and p-aminophenylmercuric acetate (APMA) are used to activate latent enzymes.

TIMPs are quantitated by immunoassay (EIA) [28] using kits #QIA31-1EA (TIMP-1) and #QIA40-1EA (TIMP-2) purchased from Bio Trak (Oncogene Research Products, Cambridge, MA).

6.A.5.Zymography and Immunoprecipitation.

Gelatin zymography follows a modified procedure of Herron et al. [29] for detecting picograms of MMP-2 & -9 and nanograms of other MMPs and proteases. SDS-PAGE is performed in 7.5% or 10% polyacrylamide (30) containing 0.33 mg/ml gelatin (or other substates i.e. casein, soluble elastin or transferrin. The gels are then rinsed twice in 0.25% Triton X-100, and incubated (18 h, 37° C in Tris-NaCl-ZnCl₂ -ZnCl₂ ,3 mM phenylmethylsulfonyl fluoride (PMSF) assay buffer. Gels are stained with Coomassie blue R 250. Both latent and active forms of gelatinases or other MMPs produce clear areas in the gel. The relative amounts of enzymes are quantitated using densitometry analysis of gels (and also dried gels in membranes).The imager (Ultra Violet Products (UVP), Upland, CA) and GelBase/GelBlot Pro Software are used to scan and quantitate 250-500 gels generated per year.

Tissue extracts and filtered media from cell lines (diluted or concentrated) are immunoprecipitated with rabbit anti-MMP(s) or anti-TIMP(s) IgG(s) using protein A-agarose suspensions. Blanks are prepared with specific IgG alone, preimmune serum with enzyme fraction, and enzyme with protein A gels but no IgG. After the reacted agarose gels are washed, the immune complexes [31] dissolved in sample buffer and analyzed by zymography for specific enzyme activity or for TIMPs by reverse zymography or for proteins by SDS-PAGE. By this method, 90-95% of the antigen present is immunoprecipitated.

6.A.6. Reverse zymography: A modified method of Heron et al [30] is used to detect the TIMPs. Recently, a kit is available to perform reverse zymography of tissue extracts and conditioned media from University Technologies International Inc., Alberta, Canada. The kit provides standards of TIMP-1, -2 and -3 and the media containing the enzymes. Conditioned media (or extract) is fractionated by SDS-PAGE [30] electrophoresis using 12.5% acrylamide, 0.75 mg/ml gelatin solution and the supplied media (0.1 ml) containing enzymes. After washing with 2.5% Triton X-100, the gel is incubated in Tris buffer (37°C, and minimum gentle shaking) and stained with Coomassie blue solution to reveal cleared and uncleared area of the gelatin in the gel. Uncleared blue staining areas are revealed only if TIMPs are present. This is a very sensitive method revealing as little as 2 ng of TIMPs in cell conditioned media where other protein production is limited. This kit does not provide calibrated TIMP standards.

6.A.7. Quantitation of TIMP-1 by Chemiluminescence Western Blot: Quantitation of TIMP-1 in breast tissue extracts was carried out by chemiluminescent Western blot analysis using HRP-labeled second antibody. Equal quantity of cellular protein from each tissue extracts were electrophoresed in a 12% polyacrylamide gel. After electrophoretic transfer to a nitrocellulose membrane, the blocked (1% milk protein) membrane was stained with primary (rabbit anti-TIMP-1 human, 0.5 µg/ml or 1:700) antibody. Washed membrane was then stained with HRP-labelled second antibody followed by Western Blot Chemiluminescence reagent (NEL-102, Dupont, Boston MA) and exposed to Dupont Reflection™ autoradiography film for 10-30 seconds. The film was scanned by an imager and the TIMP related bands were quantitated using the GelBase/GelBlot Pro Software (Ultra Violet Products (UVP), Upland, CA). Quantitated TIMP-1 standard was included in each gel.

6.A 8.. RNA extraction and reverse transcription polymerase chain reaction (RT - PCR analysis).

Tissues aliquots or confluent cells (fresh) rapidly frozen in liquid nitrogen and stored at -70°C are used. Total cellular RNA is isolated by a single-step acid guanidium thiocyanate-phenol-chloroform RNA extraction method [32] using TRI REAGENT^R (Molecular Research Center, Cincinnati, OH, USA) according to manufacturer's protocol. Five micrograms of total RNA are reverse-transcribed by using a SuperScript™ kit, Cat# 18089-011 (GIBCO BRL, Gaithersburg, MD) with random primers. To amplify metalloproteinases (MMP-2, MMP-9, MMP-1), and TIMP-1 or TIMP-2, one-tenth of the cDNA pool is subjected to subsequent PCR amplification by adding 2.5 U of Taq DNA polymerase (GIBCO, Cat# 18038-042) to 50 pmol of 5'- and 3'- sequence specific oligonucleotide primers, dNTP (10 mM, Gibco BRL Cat#18427-013) in a buffer containing 10 mM Tris-HCL, 1.5 mM MgCl₂, 50 mM KCL, and 0.1 mg/ml gelatin, pH 8.3, in 100 µl volume. The mixture is overlaid with mineral oil and then subjected to amplification in a Model 2400 Perkin-Elmer thermal Cycler (Perkin Elmer, Norwalk, CT). A standard program of 30 cycles is used, consisting of denaturing at 94°C for 90s, annealing at 58°C for 90s, and extension at 72°C for 3 min, and a final extension at 72°C for 5 min. Expression of GAPDH, a housekeeping gene, was also analysed as an internal standard [33,34]. The standard method where each MMP(s),

TIMP(s) and GAPDH (housekeeping gene) from one breast tissue was performed singly leading to a semi quantitative expression and the results from various breast tissues were reported in the annual report of 1997.

For more accurate quantitation of gene expression of enzymes and inhibitors in breast tissues, we have included GAPDH specific primers in each MMP or TIMP RT-PCR reaction so that each enzyme or inhibitor values can be normalized to GAPDH values for the final gene expression. Modified conditions for above standard RT-PCR method has been developed to include specific primers for both GAPDH and MMP(s) or TIMP(s) in the same RT-PCR reaction tube. The conditions of annealing temperature and number of cycles for MMP-9, MMP-2 and TIMP-1 are shown in **TABLE 1**. Briefly, PCR reaction is carried out using MMP or TIMP specific primers for 5 cycles, annealing temperature at 65°C, then addition of GAPDH specific primers and PCR reaction carried further for 35 cycles.

TABLE 1. PCR Reaction - Modification conditions:

Gene	Mgcl ₂	Specific primers	Cycles	Annealing temp	GAPDH (ng) primers added	Annealing temp	Cycles
MMP-9	1.6 mM	150 ng	5	65° C	130 ng	65° C	35
MMP-2	1.5 mM	160 ng	5	65° C	130 ng	65° C	35
TIMP-1	1.5 mM	150 ng	2	65° C	130 ng	65° C	35

Primers [33] for MMP-9, MMP-2, MMP-3, MMP-1 (collagenase 1), TIMP-1, TIMP-2 and GAPDH^b were kindly provided by Dr. V.H. Rao, Meyer Rehabilitation Institute and University of Nebraska Medical Center, Omaha, NE. These primers are synthesized using a DNA synthesizer (Applied Biosystems, Foster City, CA). Primer pairs for MMP-13 (collagenase-3) and GAPDH^a are synthesized using a DNA synthesizer (Perkin Elmer model 374 (Applied Biosystems, Foster City, CA). The primer sets listed below produced one PCR product band. Amplified PCR products are identified by electrophoresis of 5 - 10 µl sample aliquotes on 1.5% agarose gel stained with ethidium bromide, visualized by UV transillumination and photographed. Semiquantitation of MMPs and TIMPs are performed using densitometry and comparison to GAPDH (internal standard) from the same sample after using an image analysis system (Ultra Violet Products (UVP) and GelBase/GelBlot Pro Software, CA). The PCR procedure is repeated at least two or three times for each sample. Prime sequences for MMPs, TIMPs and GAPDH are recorded in **Table 2**. Sets of primers are used as described previously [35,36].

Table 2. Primers for RT-PCR analysis

Genes		Sequence	Product (bp)
MMP-2	Antisense	5'-GCA GCC TAG GGA GTC GGA TTT GAT G-3'	480
	Sense	5'-CCA CGT GAC AAG CCC ATG GGG CCC C-3'	
MMP-9	Antisense	5'-GTC CTC AGG GCA CTG CAG GAT GTC ATA GGT-3'	640
	Sense	5'-GGT CCC CCC ACT GCT GGC CCT TCT ACG GCC-3'	
MMP-1	Antisense	5'-TTC CAG GTA TTT CTG GAC TAA GT-3'	185
	Sense	5'-ATT GGA GCA GCA AGA GGC TGG GA-3'	
MMP-3	Antisense	5'-TTC TAG ATA TTT CTG AAC AAG G-3'	155
	Sense	5'-GCA TAG AGA CAA CAT AGA GCT-3'	
MMP-13	Antisense	5'-GAA CAG CTG CAC TTA Y-3'	134
	Sense	5'-TCA TGA CCT CAT CTT C-3'	
GAPDH ^a	Antisense	5'-TGA TTT TGG AGG GAT CTC GC-3'	230
	Sense	5'-ACG CAT TTG GTC GTA TTG GG-3'	
GAPDH ^b	Antisense	5'-GGA TCT CGC TCC TGG AAG ATG GTG ATG GG-3'	237
	Sense	5'-GGT GAA GGT CGG AGT CAA CGG ATT TGG TCG-3'	
TIMP-1	Antisense	5'-GGC TAT CTG GGA CCG CAG GGA CTG CCA GGT-3'	551
	Sense	5'-TGC ACC TGT GTC CCA CCC CAC CCA CAG AGC-3'	
TIMP-2	Antisense	5'-GGA AGC TTT TAT GGG TCC TCG ATG TCG AG-3'	590
	Sense	5'-CCG AAT TCT GCA GCT GCT CCC CGG TGC ACC CG-3'	
MMP-14	Antisense	5'-CGC TAC GCC ATC CAG GGT CTC AA-3'	496
	Sense	5'-CGG TCA TCA TCG GGC AGC ACA AA-3'	

6.A.9. Explant cultures and cell lines: Tissues either fresh or frozen in 5% dimethyl sulfoxide are obtained according to State and Federal regulations from the Tissue Procurement Laboratories, Sylvester Cancer Center, University of Miami. Neoplastic tissues are obtained as chips or pieces and classified for tumor grades by the University of Miami Pathology Reference Service.

Tissues are collected under sterile conditions, minced into pieces, rinsed (PBS), seeded [37] in T25 flasks and cultured in MEGM - epithelial growth medium (Cat#CC-3051, Clonetics Corp, San Diego, CA) containing 10% fetal calf serum for 24-48 hours to maintain the epithelial cell growth. Once the growth is established, the cells are

washed with serum free MEGM for further 24, 48 or 72 hours. The conditioned medium (CM) is collected, filtered and stored in aliquots at -70°C until used for enzyme and TIMP analyses.

Explant cultures are also setup using fibroblast growth medium (FGM, Cat#CC-3131, Clonetics Corp, San Diego, CA). This medium sustains the growth of only fibroblasts from the tissue explants. The fibroblasts are expanded similar to epithelial cultures and the serum free conditioned media are filtered and stored at -70°C . The epithelial nature of the cell monolayer is confirmed by immunocytochemical staining, using an anti-cytokeratin monoclonal antibody specific to cytokeratin 8 and 18 (CAM 5.2, Becton-Dickinson Immunocytometry Systems, San Jose, CA). Greater than 90% of the cells should stain positive in randomly selected microscopic fields under observation [37].

6.B.RESULTS:

6.B.1. Quantitation and characterization of MMPs and TIMPs in breast tissue: Of 150 breast tissue samples that were processed for extraction of MMPs and TIMPs, only 122 breast tissue samples correlated well with pathology reports. Corresponding blocks were also available for immunohistochemistry analyses. This part of the study is complete. There are sufficient samples in each group to evaluate the results with confidence.

It was necessary to use three extraction procedures (Triton X 100, buffered 1 M guanadine and 0.1% SDS) for each tissue to . Triton extracts remove soluble proteins from the tissues and extracted only 40-60% of the MMPs. Buffered 1 M guanadine showed remaining enzymes present in the tissues as no enzymes were noted in 0.1% SDS extracts. SDS contained bulk of TIMP-3 as revealed by reverse zymography (data presented in earlier reports). The amount of MMPs and TIMPs present in the breast tissue extracts were calculated as total units or μg of enzyme/g wet weight tissue for MMPs or μg inhibitor/g wet weight tissue for TIMPs. The results are presented in **Table 3 and 4**.

The results in **Tables 3 and 4** categorically show that there is an **imbalance** in which the destructive proteases (**MMPs**) greatly outweigh the controlling inhibitors (**TIMPs**), destroying the basement membrane constraints and thus facilitating the spread of cancer.

The results presented in **Table 3**. show the quantitation of gelatinases (**MMP-9 and MMP-2**) by zymography and enzyme substrate (tritiated gelatin) assays. **MMP-1** (interstitial collagenase) or **MMP-13** (collagenase -3) were quantitated using tritiated rat soluble collagen. **MMP-1**, **MMP-13** and **MMP-8** (neutrophil collagenase) digest the collagen in the assay system used [26]. **MMP-1** in many cases cannot be detected by gelatin zymography due to poor digestion of gelatin by **MMP-1**. The ratios of latent to active **MMP-9** and **MMP-2** were obtained from the zymographical analysis. Stromelysin-1 or **MMP-7** (matrilysin, PUMP-1) were not detected in any of the breast tissue extracts. No bands were obtained in transferrin zymography. We have not included the zymographical profiles of the breast tissues analyzed in this report but the tabulation of MMPs in **Table 3** (completed). Most zymographical profiles for each category of breast tissues were similar to those presented in the previous reports.

TABLE 3. Quantitation of Matrix Metalloproteinases--Gelatinases (A[@]+B[#]) and Collagenase (MMP-1, or -13)[^] in Human Breast Tissue Extracts[†].

Tissue	No.	MMP-2 [@] + MMP-9 [#] μg [*]	MMP-9 L/A	MMP-2 L/A	MMP-9 [‡] μg [*]	MMP-1 [^] μg [*]	Total Enzyme μg
Normal	6	0.62 ± 0.02	N/A	7.8	N/A	0.05 ± 0.01	0.67
Benign Tumor	20	2.10 ± 0.04	N/A	4.10	N/A	0.71 ± 0.03	2.81
Benign Tumor Tubular	3	3.41 ± 0.5	N/A	1.6	N/A	0.24 ± 0.02	3.65
Adeno- carcinoma GRIII	46	8.4 ± 0.10	0.92	1.6	3.5 ± 0.03	0.95 ± 0.02	9.35
GRII	29	3.2 ± 0.07	1.85	2.55	0.59 ± 0.02	0.74 ± 0.1	3.94
Lobular carcinoma Infiltrating	5	2.9 ± 0.04	0.71	2.05	0.66 ± 0.01	0.7 ± 0.6	2.95
<i>In Situ</i> ductal carcinoma	6	1.05 ± 0.03	1.4	4.18	0.35 ± 0.02	0.15 ± 0.01	1.20
<i>In-Situ</i> lobular carcinoma	4	1.35 ± 0.05	1.1	4.5	0.52 ± 0.03	0.75 ± 0.04	2.10
Colloidal carcinoma	3	1.9 ± 0.03	1.1	1.9	--	--	--

Gelatinase A =MMP-2[@] + gelatinase B =MMP-9[#], Total Units = latent + active enzyme from breast extracts.
[†] 0.25% Triton extracts + 1M GuHCl extracts.

One enzyme unit = 1μg of substrate digested /min at 37° C for gelatinases & at 30° C for collagenase.

* Values expressed as μg/g wet weight breast tissue. -- units of enzyme activity converted to μg.

Values are given as mean ± SEM (standard error of the mean).

L/A = Latent enzyme/Active enzyme ratio.

[‡] Amount of MMP-9 enzyme from total gelatinase - calculated from zymography - imager analysis.

Adenocarcinoma = Infiltrating Ductal Carcinoma - Nuclear Grade III or Nuclear Grade II.

[^] Interstitial collagenase - MMP-1 or MMP-13 or both.

Biochemical quantification of MMPs showed an overall increase in all types of MMPs in cancer tissues (Table 3). **MMP-9** was the **key MMP**, it was present at levels **0.59 μg** (29.5.units)/g wet weight tissue in *in situ* iductal to **3.5 μg** (175 units)/g wet weight tissue in infiltrating ductal carcinoma (grade III). **Lobular** carcinoma also showed high levels of **MMP-9**. Zymography also shows a fraction of MMP-9 and MMP-2 in their active forms. There is a corelation of constitutively expressed MMP-1 (0.05 - 0.95 μg /g wet weight tissue) in cancer tissues. Constitutively expressed **MMP-2** also increases.

It was difficult to find in literature, the analysis of benign breast tissues by zymography. The levels of **MMP-9** are highest in infiltrating and poorly differentiated ductal carcinoma (Grade III) breast tissue extracts than any of the other carcinomas . Results in **Table 3** clearly show that **MMP-9, 92 kDa type IV collagenase or gelatinase B** is found in varying amounts in cancer tissues when compared with normal and benign breast tissues.

MT1-MMP (membrane type metalloproteinase) was not quantifiable by zymography. Confocal analysis using anti-MT1-MMP) IgGs (kindly provided by Dr. G. Goldberg, Washington university, St. Louis), showed that MT1-MMP was seen only in breast cancer tissues. This evaluation was reported last year (Progress Report 1996).

The breast tissue extracts were scanned for **TIMPs** by reverse zymography. **TIMP-1, -2 & -3** are detected in ng quantities. The tissue extracts are fractionated by SDS-PAGE (12,5% acrylamide, gelatin and enzyme media) electrophoresis The uncleared blue bands are due to the TIMPs present in the extracts or conditioned media. All 3 TIMPs are present in different amounts in the breast tissue extracts Of the three different extracts, only 1M GuHCl and SDS extracts can be scanned for TIMPs. The SDS extraction step is necessary to remove TIMP-3 entrapped in the matrix of the tissue. The Triton extracts contain other soluble proteins and it becomes difficult to distinguish the TIMP bands from the regular same molecular weight protein bands that did not diffuse out of gel during incubation. Conditioned cell media can be successfully quantitated for TIMPs. Protein profiles by SDS-PAGE electrophoresis were obtained parallel with reverse zymography of Triton and 1 M guanadine extracts. the rational of scanning both gels i.e. reverse zymography and protein gels to remove the density of protein bands to give us the amounts TIMPs present in each breast tissue. This was a futile attempt. The smaal amounts of TIMPs present in the tissues is overwhelmed by larger proportion of the same molecular weight proteins.

Western blot method was next cosidered to quantitate the reacted TIMP bands with rabbit **anti-TIMP-1 & -2** using sensitive chemiluminesce method (see Methods section fro details). This method is frequently used in quantitating TIMPs in cell conditioned media. Dialyzed breast tissue extracts were eletrophoretically separated transfered to nitrocellulose paper. The paper was stained with primary and secondary antibodies and TIMP bands to be revealed on X-ray film. This method was not as sensitive as reverse zymography or ELISA kits. A faint band could be seen with 60 ng of TIMP-1. Other protein bands at higher molecular weight also reacted showing smear upto just above TIMP-1 band (result not shown).

Table 4. Amounts of Tissue Inhibitors of Metalloproteinases (TIMPs -1, -2) in dialyzed Triton X-100 & 1M GuHCl extracts of Breast Tissues.

Tissue	No.	TIMP-1 μg*	TIMP-2 μg*
Normal	5	2.6	1.9
Benign	15	2.1 ± 0.04	3.5 ± 0.03
Adeno carcinoma Grade III	32	0.14 ± 0.05	1.35 ± 0.15
Grade II	18	0.14 ± 0.05	2.10 ± 0.04
<i>in situductal</i> carcinoma	5	1.5 ± 0.12	2.9 ± 0.09

*Breast tissue extracts (Triton and GuHCl) are analyzed for TIMP-1 and TIMP-2 (μg/g wet weight tissue) by TIMP-1 (Cat # QIA31) & TIMP-2 (CAT#QIA40) ELISA kits (BIOTRAK) purchased from Calbiochem (Oncogene Research Products).

For TIMP-1, the diluted extracts showed different values to those obtained for undiluted extracts and these values were higher than those obtained from reverse zymographical-imager analysis.

Caliberated TIMP standards of known values (ng) are used.

Adenocarcinoma = Infiltrating ductal Carcinoma - Nuclear Grade II or III

We next proceeded to quantitate the dialyzed breast tissue extracts by ELISA systems for TIMP-1 and TIMP-2 purchased from Oncogene Research Products (Calbiochem, Cambridge, MA). This procedure was not satisfactory to quantitate absolute amounts of TIMPs present in the tissue. Solutions containing TIMPs standards showed a good correlation upon dilution of the solutions. However, this was not the case with tissue extracts. Dilutions of breast extract solutions did not match with undiluted dialyzed breast tissue extracts. So we analyzed some of the tissue extracts (undiluted) by ELISA and results are reported in **Table 4**. This Table shows (although may be inaccurate in absolute amounts) however, a trend of lower amounts of TIMP-1 and TIMP-2 ($\mu\text{g/g}$ wet weight tissue) found in **50** cancer tissues compared to the higher amount found in normal (**5** samples) and benign (**15** samples) tissue.

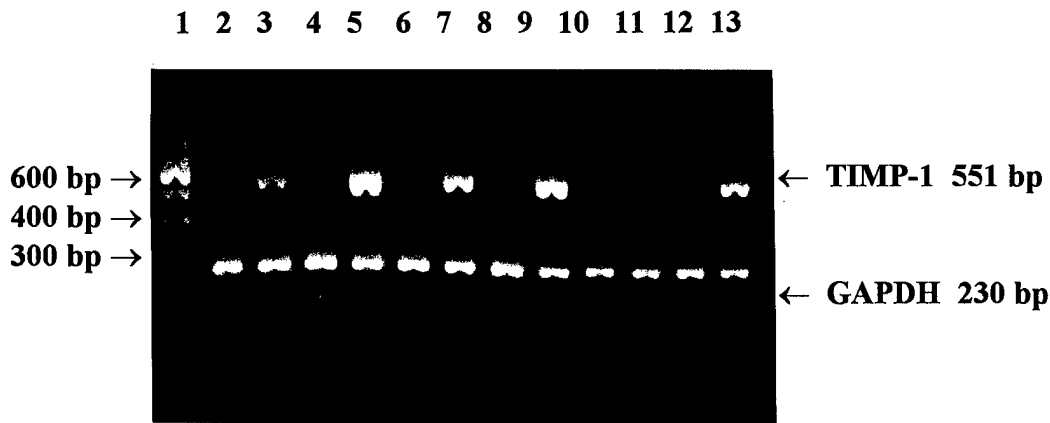
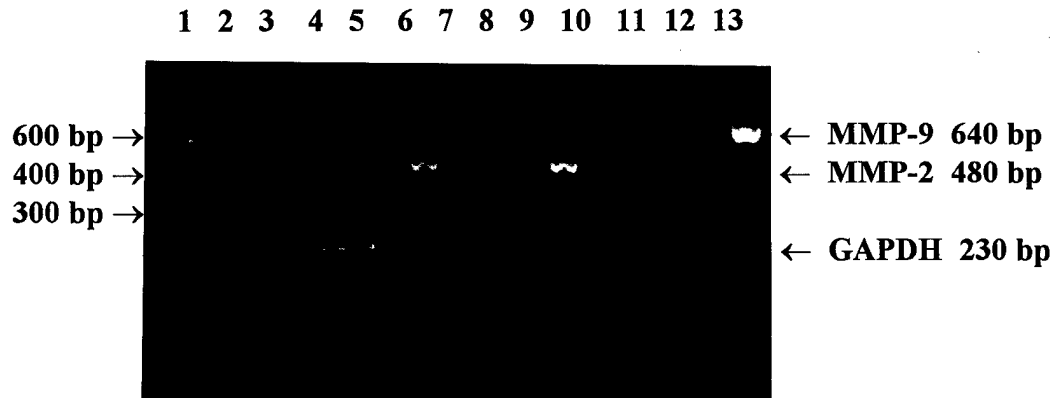
The results shown in **Tables 3 and 4** clearly indicate that the amounts of MMPs present in cancer breast tissues (10 μg , Grade III cancer) has available only **1.5 -3.5 μg** of **TIMPs** for arresting the destruction of tissue by MMPs. There are other MMPs and TIMPs present in the tissue and their quantitation has not been accounted in this study..

6.B.2. Immunohistochemical evaluations of breast tissue:

Immunohistochemical analyses of over 100 breast tissue samples for various MMPs and TIMPs were carried out over last four years. The results were presented in the Reports of 1995, 1996 and 1997. We have stained many more samples this year and the results were more or less similar to those reported in previous reports and are not included in this report. In all additional 20 breast tissue samples representing over 200 slides were processed in 1997-1998. We are in the process of scanning over 1000 slides to obtain slides that best represent each type of breast cancer. These slides will then be used for the manuscript in preparation. The manuscript will correlate the immunohistochemical analysis with zymographical profiles of the same breast tissue.

Each tissue section is photographed for a slide using the microscope available in the Pathology Department, University of Miami. The slides are carefully evaluated with Dr. Scott Sittler, Assistant Professor, Department of Pathology, University of Miami Fl and recorded using grading system of 1-4 with + sign against each number as 1⁺, 2⁺ to 4. The terminology of negative or positive to IgGs are also used besides the grading system. All IgGs used in this study were monospecific and evaluated periodically by western blots.

Eleven types of breast tissues showing normal, benign and various types of carcinomas have been stained for various MMPs and TIMPs. The staining of normal breast tissue showed virtually very little staining with anti-MMP-9 and shows staining only with MMP-2, MMP-1, TIMP-1 and TIMP-2. No staining was observed with MMP-3 or MMP-7. Staining of benign, fibroadenoma breast tissue sections revealed virtually no staining with anti-MMP-9 IgGs, but stains heavily with anti-MMP-2. **Infiltrating and poorly differentiated ductal carcinoma, Grade III (IDC)** stains heavily with anti-MMP-2, -MMP-9, -MMP-1 and yet show only weak staining with anti-TIMP-1. **This suggests an imbalance between MMPs and TIMPs in IDC Grade III infiltrating ductal carcinoma.** Medullary, and in situ lobular sections stain highly for enzymes overall and has weak staining for TIMP-1. Papillary carcinoma sections did not stain for

A: Expression of TIMP-1 & GAPDH in Breast Tissues.**B: Expression of MMP-9, MMP-2 & GAPDH in Breast Tissues.****FIGURE 1. Expression of MMP-2, -9, TIMP-1 & GAPDH in breast tissues.**

cDNA was prepared from breast tissues and RT-PCR was performed using specific primer pairs for each MMP or TIMP-1 & GAPDH in the same reaction tube (details Table 1 in the Methods section). The amplified products were separated on 1.5% agarose gels and stained with ethidium bromide. A: Expression of TIMP-1 & GAPDH. B: Expression of MMP-2, or MMP-9 & GAPDH. Lanes 1A & 1B- standards; lanes 2A, 3A, 2B, 3B, & 4B showing GAPDH & low expression of TIMP-1 & expression of MMP-2 & MMP-9 in infiltrating lobular carcinoma; lanes 4A, 5A, & 5B, 6B, & 7B showing GAPDH, higher expression of TIMP-1 & virtually no expression of MMP-9 in benign breast tissue; lanes 6A, 7A, showing GAPDH & reasonable expression of TIMP-1 in Grade II, infiltrating ductal carcinoma; lanes 8A & 9A, showing GAPDH & TIMP-1 in *in situ* lobular carcinoma; lanes 10A & 11A, 11B, 12B & 13B showing GAPDH, lower expression of MMP-2, highest expression of MMP-9 & virtually no expression of TIMP-1 in Grade III, infiltrating & poorly differentiated ductal carcinoma; lanes 12A, 13A, 8B, 9B & 10B showing expression of MMP-2 & comparatively lower expression of TIMP-1 in *in situ* ductal carcinoma. RT-PCR analysis were carried out in over 80 breast samples & a few representative results are presented in Figures 1 & 2.

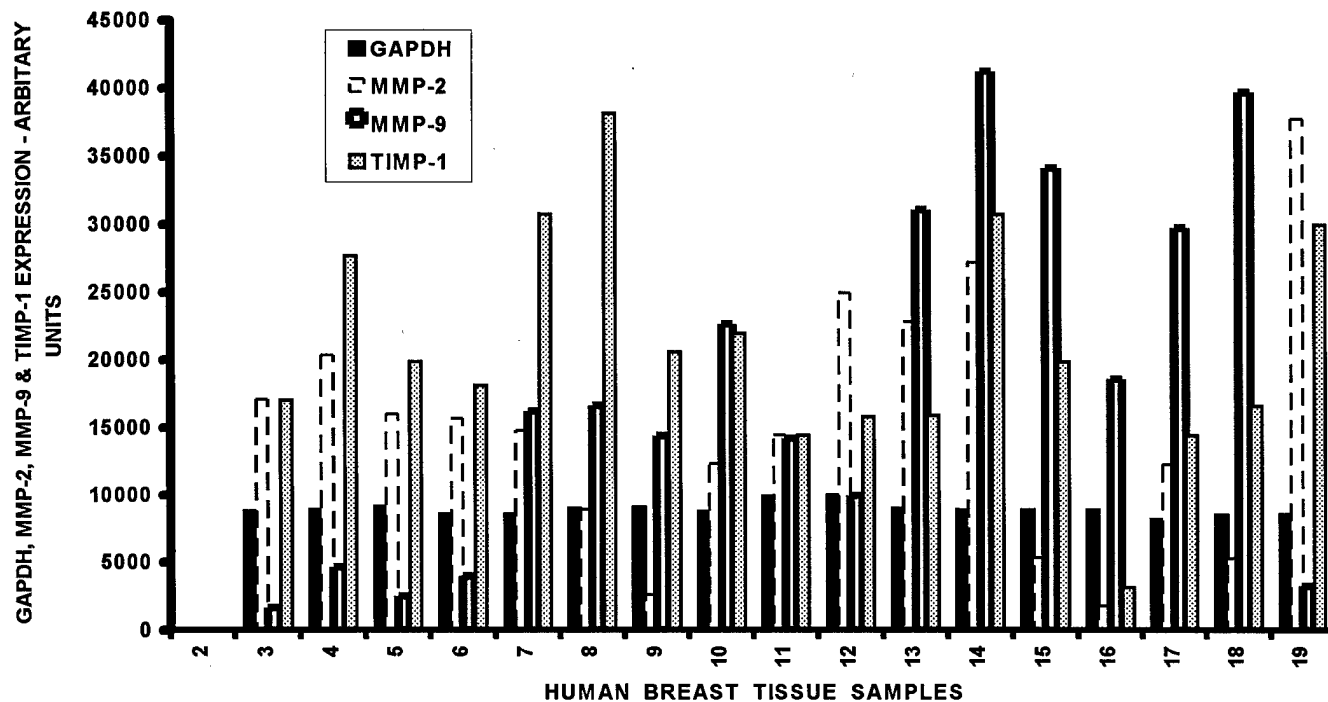


Figure 2. Quantitative Expression of GAPDH, MMP-2, MMP-9 & TIMP-1 in Breast Tissue Samples

Expression of GAPDH (230 bp fragment), MMP-2 (480 bp fragment), MMP-9 (640 bp fragment) & TIMP-1 (551 bp fragment) as determined by PCR. cDNA was prepared from breast tissues and RT-PCR was performed using specific primer pairs for each MMP or TIMP-1 & GAPDH in the same reaction tube (details Table 1 in the Methods). The amplified products were separated on 1.5% agarose gels and stained with ethidium bromide. The bands thus obtained were scanned by an imager and analyzed by GELBASE/GELBLOT PRO software (UVP Products). Units of peak area are arbitrary. However, the values were normalized to GAPDH units for MMPs & TIMP-1 in each breast tissue. Lanes 3, 4, 5 & 6 showing very low expression of MMP-9 in benign breast tissues; lanes 7 & 8 showing higher expression of MMP-9 & TIMP-1 in *in situ* ductal carcinoma; lanes 9 & 10 showing lower expression of MMP-2 & higher expression of MMP-9 in *in situ* lobular carcinoma; lanes 11 & 12 showing higher expression of MMP-2 & MMP-9 & comparatively lower expression of TIMP-1 in Grade II, infiltrating ductal carcinoma; lanes 13, 14 & 15 showing highest expression of MMP-9, MMP-2 & comparatively lower expression of TIMP-1 in Grade III, infiltrating & poorly differentiated ductal carcinoma; lanes 16 & 17 showing lower expression of TIMP-1 in infiltrating lobular carcinoma; Lane 18 showing highest expression of MMP-9 only in metaplastic poorly differentiated carcinoma and lane 19 showing lower expression of MMP-9 in benign, fibrocystic breast tissue.

anti-MMP-9. The results also indicate that in IDC tissue sections viewed under microscope at 100 X show that tumor cells indeed reveal cytoplasmic staining only with anti-MMP-9. MMP-2 staining of the same section shows the staining of stroma and very few tumor cells. Immunohistochemical analyses categorically show that TIMP(s) staining is weak in carcinoma tissues while they stain highly with gelatinases and MMP-1. Again no specific staining was observed with anti-MMP-3 or MMP-7 in breast carcinoma tissues.

6.B.3. Reverse transcription polymerase chain reaction (RT-PCR) analysis. Detection of low-abundance mRNAs by RT-PCR has now become a standard technique to determine gene expression in tissues. To determine relative or absolute copy numbers of specific mRNAs is difficult without internal standards as control for sample to sample variation. We examined and quantitated the expression of MMPs and TIMPs in breast tissue samples using RT-PCR analyses. Northern blot analysis were not successful using RNA isolated from various breast tissues. Freshly harvested breast tissue and cells from different cell lines showed the expression of MMPs and TIMPs by northern blots. The feasibility of acquiring 100 plus fresh samples for this multipronged study was impossible. We therefore traded my antibodies for MMPs and TIMPs with purified specific primer pairs from Dr. V.H. Rao. The primers selected for amplification (see **Table 2**) have been shown to produce a single product of the appropriate size for MMP-9, MMP-2 [33], TIMP-2, TIMP-1 and GAPDH, a house keeping gene [33]. We were successful in processing breast tissue samples.

The results obtained by the standard RT-PCR method were semi quantitative and the results were reported in 1997 report. There seemed to be a variation in the expression of GAPDH. Relationship between GAPDH and MMPs and TIMPs remained questionable.

Modification of standard RT-PCR method was necessary to include GAPDH primers in each MMP and TIMP primers in the same reaction tube (see Methods section). This modification would allow the normalization of MMPs and TIMPs to GAPDH expression in each tissue. We processed over 50 breast tissue samples containing benign and various carcinoma samples. The representative results reported in **Figures 1 & 2..**

. RT-PCR method proved to be useful and at least we can utilize the extracted RNA's from one to five year frozen breast tissue samples that we have processed for zymography and immunohistochemical analysis. Exact number of expressed copies can be achieved by competitive RNA templates for detection of MMPs and TIMPs by RT-PCR procedure described by Tarnuzzer et al. [38]. This method will eliminate sample to sample variation and several MMPs and TIMPs can be expressed from as little as 4 µg of total RNA. This method is time consuming in processing several breast samples.

The results are reported in **Figures 1 & 2**. So far, we have analyzed over 50 breast tissue samples by the **modified RT-PCR method**. **Figure 1** shows the expression of **GAPDH, MMP-2, MMP-9 and TIMP-1**. The corresponding bands were then analyzed using imager and quantitated by **GELBLOT/GELPRO software** and values are reported in **Figure 2**. Units of peak are arbitrary. Normalisation of MMPs and TIMPs expression to GAPDH expression in the same breast tissue eliminates the variation in absolute values. Lanes **3, 4, 5 & 6** showing **very low** expression of **MMP-9** in **benign** breast tissues; lanes **7 & 8** showing **higher** expression of

MMP-9 & TIMP-1 in *in situ* ductal carcinoma; lanes 9 & 10 showing lower expression of MMP-2 & higher expression of MMP-9 in *in situ* lobular carcinoma; lanes 11 & 12 showing higher expression of MMP-2 & MMP-9 & comparatively lower expression of TIMP-1 in Grade II, infiltrating ductal carcinoma; lanes 13, 14 & 15 showing highest expression of MMP-9, MMP-2 & comparatively lower expression of TIMP-1 in Grade III, infiltrating & poorly differentiated ductal carcinoma; lanes 16 & 17 showing lower expression of TIMP-1 in infiltrating lobular carcinoma; Lane 18 showing highest expression of MMP-9 only in metaplastic poorly differentiated carcinoma and lane 19 showing lower expression of MMP-9 in benign, fibrocystic breast tissue.

The development of the RT-PCR procedures provide facile methods for investigating the expression of MMPs and TIMPs.

Using standard RT-PCR procedure and specific primer pairs for MMP-1 (collagenase -1) showed a single band in most breast tissues for the expression of MMP-1 of 185 bp. With MMP-13 specific primers, there were two distinct bands suggesting changes in annealing temperature and number of cycles. This part of the project is in progress. Preliminary results show that MMP-13 is mainly expressed in metaplastic and Grade III infiltrating ductal carcinoma tissues. MMP-1 is expressed in all breast tissues. MMP-1 expression seems constitutive.

6.B.4. Tissue explant cultures: We obtained some fresh breast tissues consisting of one benign, one IDC and one *in situ* ductal carcinoma tissues to evaluate the explant cultures. We were successful in obtaining and sustaining the growth of epithelial cell line with the prostate tissue explants [37]. The epithelial cell line growth was expanded in 6-well culture dishes with F-12 media containing 10% fetal calf serum for 24 hours. After washing cells with PBS the cells were maintained in serum free media for further 24, 48 and 72 hours. The media were collected, filtered, concentrated and analyzed for MMPs and TIMPs by zymography, and reverse zymography. Very faint bands of MMP-9 were seen by zymography (result not shown) in 72 hour culture. More tissue than what was seeded may be needed to obtain decent number of cells. We also attempted to obtain a fibroblast cells from tissue explant cultures using FGM media. The fibroblastic cells were contaminated with fungi and were discarded. Further attempts to obtain expandable epithelial and fibroblastic tissue explant culture will be repeated in the next few months. An extension of the granting period without further monies is granted by DOD administrative office. Successful explant cultures of breast epithelial cells and fibroblastic cells may help to clarify the contraversial reports that stimulation of stromal fibroblasts by tumor cells produce most of the MMPs specifically MMP-9. Little success in epithelial explant culture did show the production of MMP-9.

(7) CONCLUSIONS:

MMPs and TIMPs represent a class of metalloproteases and their tissue inhibitors secreted by various types of cells, including epithelial, fibroblasts, and macrophages. MMPs have been implicated in degradation of basement membrane during cancer invasion and metastasis. Their activity is controlled, in part, by natural inhibitors (TIMPs). The imbalance created in the secretion of their tissue inhibitors (TIMPs) has been implicated by us for prostate cancer [37].

We have analyzed over 150 biopsied breast tissues containing normal, benign, infiltrating ductal carcinoma (Grade I and II), lobular carcinoma *in situ* ductal and lobular, papillary carcinoma, medullary carcinoma and demonstrated that biopsied breast tissues is suitable to carry out a multi pronged study to obtain data using biochemical, immunohistochemical and molecular biological evaluations of MMPs and TIMPs.

This multipronged study points to the role of MMP-9 as the key enzyme that is elevated in cancer tissues. This finding is in agreement with RT-PCR, zymography and immunohistochemical findings. Role of MMP-9 in cancer has been implicated by several workers [9, 20, 41]. Immunohistochemical analysis showed that in many cancer tissues, we observed the red signal of antigen (MMP-9) is mainly in the cytoplasm of the tumor cell clusters. Higher magnification X 100 clearly showed the red signal of MMP-9 in the cytoplasm of tumor cells and not in the stroma surrounding tumor cells. Cellular localization of the production of MMP-9 or Mmp-2 mRNA or immunoreactivity in breast cancer or other cancers has been a source of controversy and speculation. [2,3]

Besides elevated levels of MMP-9, several other MMPs were also elevated in breast cancer tissues. Levels of MMP-1 although low in amounts showed an increase from 0.05 - 0.95 $\mu\text{g/g}$ tissue in cancer tissues. The activity range of MMP-1 (0.95 μg) can disrupt the extracellular matrix by digesting the collagen molecules present. The tumor cells come in contact with growth factors present in the matrix and support the growth of tumor cells [9]. MMP-1, MMP-8 and MMP-13 all digest the collagen fibres and our assay method for collagenase quantitates all three types of collagenase. We hope to use RT-PCR to quantitate the three collagenases in the coming months. We have not been able to correlate the MMP-1 activity profile with several of the immunohistochemical evaluations of tissues using anti-MMP-1 IgGs. We suspect that MMP-8 and MMP-13 collagenase-3 may be present in higher amounts than MMP-1 in the cancer tissues. Collagenase-3 (MMP-13) has been implicated in breast cancer tissues [18].

The breast tissue extracts showed the presence of different types and levels of TIMPs by reverse zymography. We were able to detect TIMP-1, TIMP-2 and TIMP-3 (data in earlier reports). We have quantitated the TIMP-1 and TIMP-2 by ELISAs purchased from Oncogene Research Products. The results for TIMP-1 and -2 by ELISA method showed categorically that the TIMPs were indeed in lower amounts than the sum of some of the enzymes that were quantitated.

The breast tissue sections stained with anti-TIMP-1, anti-TIMP-2 and anti-TIMP-3 IgGs showed weak staining of stromas. Some cancer tissues showed staining around the tumor cell clusters. The RT-PCR also exhibited comparatively low expression of TIMP-1 and combining these findings from multi pronged study shows that there is an imbalance in which the destructive proteases greatly outweigh the controlling

inhibitors, facilitating the spread of cancer. These findings strongly suggest that the basement membrane underlying breast epithelium probably undergoes rapid turnover due to matrix degrading enzymes secreted by various resident cells in the breast tissue [2]. Enhanced RNA expression of TIMP-1 in human breast cancer has been reported [39]. Our multipronged study categorically shows lower expression of TIMPs.

The measurement of mRNA levels of TIMPs and MMPs by Northern blots has been frustrating. However, RT-PCR method has been established in my laboratory and analysis of over 50 breast tissues for the expression of MMPs and TIMPs in these tissues has been achieved.

Meaningful statistical analysis of 150 breast samples have been completed. Over 1000 slides for immunohistochemistry have been reviewed. Completion of RT-PCR analysis for MMP-1 and MMP-13 are now feasible in the next few months. Storage of fresh frozen samples over five years and the availability of information from Florida Tumor Registry of follow-ups of patients will allow us to fine tune the collected information. Our study definitely suggests that an inhibitor specially designed to knock out the MMP-9 enzyme activity may arrest the invasiveness and metastatic potential of breast cancer cells.

One full refereed article, two abstracts, and one short report has been generated from the research project. In the next few months, the completed findings will generate two full manuscripts. (1) Immunohistochemical and zymographical evaluations of MMPs and TIMPs in breast cancer. (2) mRNA levels of MMPs and TIMPs and their cellular location in breast cancer.

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STATEMENT OF WORK

Role of Matrix Metalloproteinases and Their Tissue Inhibitors in Human Breast Adenocarcinoma

Task 1. Additional specimens have been obtained and over 150 breast tissue samples have been processed to complete the study.

a. Year 5: Preparation of manuscripts (possibly 2) from the completed study of the quantitation of enzymes and inhibitors (MMPs and TIMPs) by zymography, immunohistochemical analyses and gene expression by RT-PCR.

Task 2. Fresh tissues will be collected from patients for explant epithelial and fibroblast culture studies. These findings will be related to the nearly completed study of enzymes and inhibitors.

Task 3. Correlate the findings obtained from the immunohistochemical analyses, quantitation of MMPs and TIMPs and their mRNAs, pinpoint the over- or under-expression of MMPs or imbalance between the enzymes and inhibitors. Design further experiments to bolster or refute the underlying hypothesis using explant culture studies. This task will be ongoing and adequate data will probably be available in the next six months..

(9) APPENDICES

9. 1. Manuscript I

CD44_{v3,8–10} Is Involved in Cytoskeleton-Mediated Tumor Cell Migration and Matrix Metalloproteinase (MMP-9) Association in Metastatic Breast Cancer Cells

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In the present study, we have employed a unique breast cancer cell line (Met-1, which was derived from a high metastatic potential tumor in transgenic mice expressing polyomavirus middle T oncogene) to study the role of CD44 variant isoform(s) in the regulation of metastatic breast tumor cell behavior. The results of reverse transcriptase–polymerase chain reaction, Southern blot, nucleotide sequencing, immunoprecipitation, and immunoblot analyses indicated that these cells express a major CD44 isoform (molecular weight \approx 260 kDa) containing a v3,8–10 exon insertion (designated as CD44_{v3,8–10}). In addition, we have determined that CD44_{v3,8–10} binds specifically to the cytoskeletal proteins such as ankyrin. Biochemical analyses, using competition binding assays and a synthetic peptide identical to NGGNGTVEDRKPSEL (a sequence located between aa480 and aa494 of CD44_{v3,8–10}) indicate that this 15-amino acid peptide binds specifically to the cytoskeletal protein ankyrin (but not to fodrin or spectrin). This peptide competes effectively for ankyrin binding to CD44_{v3,8–10}. Therefore, we believe that the sequence ⁴⁸⁰NGGNGTVEDRKPSE⁴⁹⁴L, located at the cytoplasmic domain of CD44_{v3,8–10}, is required for the ankyrin binding. We have also detected that CD44_{v3,8–10}-containing Met-1 cells are capable of forming membrane spikes or “invadopodia” structures and undergo active migration processes. Treatments of Met-1 cells with certain agents including anti-CD44_{v3} antibody, cytochalasin D (a microfilament inhibitor), and W-7 (a calmodulin antagonist), but not colchicine (a microtubule disrupting agent) effectively inhibit “invadopodia” formation and subsequent tumor cell migration. Further analyses using zymography assays and double immunofluorescence staining indicated that CD44_{v3,8–10} is closely associated with the active form of matrix metalloproteinase, MMP-9, in a complex within “invadopodia” structures. These findings suggest that CD44_{v3,8–10} plays an important role in linking ankyrin to the membrane-associated actomyosin contractile system required for “invadopodia” formation (coupled with matrix degradation activities) and tumor cell migration during breast cancer progression. *J. Cell. Physiol.* 176:206–215, 1998. © 1998 Wiley-Liss, Inc.

CD44 isoforms belong to a family of glycoproteins that are expressed in a variety of cells and tissues (Lesley et al., 1993; Bourguignon, 1996). The external domain of certain CD44 isoforms is known to mediate both cell adhesion to extracellular matrix (ECM) components (e.g. hyaluronic acid, fibronectin, and collagen) and homotypic cell aggregation (Stamenkovic et al., 1991; Lesley et al., 1992, 1993; Underhill, 1992; Yang et al., 1994). The cytoplasmic domain of the CD44 iso-

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forms contains a specific binding region required for ankyrin binding (Bourguignon, 1996; Lokeshwar et al., 1996). Posttranslational modification of the cytoplasmic domain of CD44 by protein kinase C (Kalomiris and Bourguignon, 1989), acylation (Bourguignon et al., 1991), or GTP binding (Lokeshwar and Bourguignon, 1992) has been found to enhance the binding between CD44 and ankyrin. These observations suggest that CD44 not only functions as an adhesion protein but also may play an important role in signaling cytoskeleton-mediated cellular activities (Bourguignon et al., 1993; Bourguignon, 1996).

It is now known that all CD44 isoforms are encoded by a single gene that contains 19 exons (Screaton et al., 1992). Of the 19 exons, 12 exons can be alternatively spliced (Arch et al., 1992; Screaton et al., 1992; Tolg et al., 1993). Most often, alternative splicing occurs between exons 5 and 15, leading to an insertion in tandem of one or more variant exons within the membrane proximal region of the extracellular domain (Screaton et al., 1992). The variable amino acid sequence of different CD44 isoforms is further modified by extensive N- and O-glycosylations (Lokeshwar and Bourguignon, 1991; Bourguignon, 1996; Lokeshwar et al., 1996) and glycosaminoglycan (GAG) additions (Bennett et al., 1995). For example, one of the CD44 isoforms (so-called CD44_{v3}) contains the v3 (or exon 7) insertion that contains heparan sulfate addition sites. It has been suggested that this molecule binds a wide range of heparin binding growth factors, cytokines, and chemokines (Bennett et al., 1995).

It is clear that the mechanisms responsible for tumor progression are very complex, involving a number of biochemical and cellular events (Hung, 1988; Zetter, 1990). In human cells, matrix metalloproteases (MMPs), such as MMP-2 (72 kDa), MMP-9 (92 kDa), and MMP-3 (57 kDa), are most likely involved in the initial degradation of the basement membrane surrounding the tumor (Liotta, 1984), which is required for tumor invasion and metastasis (Boyd, 1996). Recent data indicating colocalization of MMP-2 and integrin $\alpha v \beta 3$ on the surface of invasive melanoma cells and blood vessels (Brooks et al., 1996) suggest that there is a close interaction between MMPs and certain adhesion molecules (e.g., integrins) during tumor invasion and/or metastasis. The CD44_{v3}-containing isoforms, another class of adhesion molecule, are also preferentially expressed on the surface of metastatic tumor cells during the progression of human breast carcinomas (Bourguignon et al., 1995; Iida and Bourguignon, 1995). The question of whether there is an interaction between MMPs and CD44_{v3}-containing isoforms in metastatic breast tumor cells is addressed in the present study.

We have employed a unique breast cancer cell line (Met-1, which was derived from high metastatic potential tumors in transgenic mice expressing polyomavirus middle T oncogene) to study the role of CD44_{v3}-containing isoforms in regulating metastatic breast tumor cell behavior. Using a variety of techniques (including biochemical, immunocytochemical, and molecular biological approaches), we have found that the CD44_{v3,8-10} isoform is involved in a number of metastatic tumor cell behaviors including invadopodia formation, matrix degradation, and tumor cell migration during breast cancer progression.

MATERIALS AND METHODS

Cell culture

Mammary tumor cells containing the polyoma virus middle T (PyV-MT) transgene under the transcriptional control of the MMTV LTR promoter were used to initiate a transplantable line in nude mice. The PyV-MT transgenic mammary tumor cells were obtained from mammary tumors that arose in the transgenic colony at the Institute for Molecular Biology and Biotechnology, McMaster University (Hamilton, Ontario, Canada; Dr. William J. Muller; Guy et al., 1992). Mammaries were treated with collagenase/dispase (Worthington, Freehold, NJ), and 5×10^5 cells per 100 μ l were transplanted subcutaneously as a bolus via syringe and a 25-gauge needle into the thoracic region of nude mice. The resulting high potential metastatic PyV-MT transgenic mammary tumor line, Met-1, was maintained by serial transplantation of 1-mm³ tumor segments into either subcutaneous tissue (ectopic) or intact mammary fat pads (orthotopic).

The Met-1 tumor line was dissociated after transplant generation one and plated onto T-75 flasks to develop a tissue culture line (Young et al., 1995; Cheung et al., 1997). The Met-1 cell line was cultured in high glucose DMEM supplemented by 10% fetal bovine serum, 2 mM glutamine, and antibiotics (Sigma, St Louis, MO). The Met-1 cell line is currently in passage 30.

Immunoreagents

Monoclonal rat anti-human CD44 antibody (clone 020, isotype IgG_{2b}; obtained from CMB-TECH, Miami, FL) used in this study recognizes a common determinant of the CD44 class of glycoproteins including CD44s and other variant isoforms (Chaitin et al., 1994; Iida and Bourguignon, 1995) and is capable of precipitating all CD44 variants. For the preparation of polyclonal rabbit anti-CD44_{v3} or rabbit anti-MMP-9, specific synthetic peptides (≈ 15 –17 amino acids unique for either CD44_{v3} sequences or MMP-9 sequences, e.g., the metal binding region peptide of MMP-9) were prepared by the Peptide Laboratories of the Department of Biochemistry and Molecular Biology using an Advanced Chemtech automatic synthesizer (model ACT350). Conjugated CD44_{v3} or MMP-9 peptides (to hemocyanin or polylysine) were injected into rabbits to raise the antibodies. The anti-CD44_{v3} or anti-MMP-9 sera were collected from each bleed and stored at 4°C containing 0.1% azide. Both rabbit anti-CD44_{v3} and rabbit anti-MMP-9 IgGs were prepared by using conventional DEAE-cellulose chromatography (Campbell et al., 1974) and were tested to be monospecific (by immunoblot assays).

Metabolic labeling procedures

Confluent Met-1 cells ($\approx 1 \times 10^6/60$ -mm dish) were incubated with sulfate-free Joklik medium for 6 hr, followed by (³⁵S₀₄)²⁻ labeling (300 μ Ci/ml) for 4 hr at 37°C. These radioactive sulfate-labeled cells were washed in phosphate buffered saline, pH 7.2 (PBS), and solubilized in RIPA buffer (Lokeshwar and Bourguignon, 1991, 1992; Lokeshwar et al., 1996). Subsequently, these ³⁵S-labeled cells were used for anti-CD44-mediated immunoprecipitation as described below.

Cell surface labeling procedures

Met-1 cells suspended in PBS were surface labeled by using the following biotinylation procedure. Briefly, cells (10^7 cells/ml) were incubated with sulfo-succinimidobiotin (Pierce, Rockford, IL; 0.1 mg/ml) in labeling buffer (150 μ M NaCl, 0.1 M HEPES, pH 8.0) for 30 min at room temperature. Cells were then washed with PBS to remove free biotin. Subsequently, the biotinylated cells were used for anti-CD44-mediated immunoprecipitation, as described previously (Bourguignon et al., 1997). These biotinylated materials precipitated by anti-CD44 antibody were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to the nitrocellulose filters, and incubated with ExtrAvidin-peroxidase (Sigma). After an addition of peroxidase substrate (Pierce), the blots were developed using Renaissance chemiluminescence reagent (Amersham Life Science, England) according to the manufacturer's instructions.

Immunoprecipitation and immunoblotting techniques

Unlabeled or labeled (surface biotinylated or radioactive sulfate labeled) cells were washed in 0.1 M PBS and solubilized in RIPA buffer (Lokeshwar and Bourguignon, 1991; Lokeshwar et al., 1996). The solubilized extracts were incubated with rat anti-CD44 antibody at 4°C for 15 hr, followed by incubation with goat anti-rat IgG agarose beads at 4°C for 90 min, as described previously (Lokeshwar and Bourguignon, 1991; Lokeshwar et al., 1996). The immunoprecipitates were analyzed by 7.5% SDS-PAGE followed by fluorography. In some cases, Met-1 cells were solubilized in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 buffer and immunoprecipitated using rat anti-CD44 antibody followed by goat anti-rat IgG. The immunoprecipitated material was solubilized in SDS, electrophoresed, and blotted onto the nitrocellulose. After blocking nonspecific sites with 3% bovine serum albumin, the nitrocellulose filter was incubated with rabbit anti-CD44v3 antibody (5 μ g/ml) or rabbit anti-MMP-9 antibody (5 μ g/ml) for 1 hr at room temperature, followed by incubation with horse radish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution) at room temperature for 1 hr. The blots were developed using Renaissance chemiluminescence reagent (NEN, DuPont, Boston, MA) according to the manufacturer's instructions.

Double immunofluorescence staining

To detect "invadopodia" formation (Mueller and Chen, 1991; Monsky et al., 1994), Met-1 cells were first washed with PBS (0.1 M phosphate buffer, pH 7.5, and 150 mM NaCl) buffer and fixed by 2% paraformaldehyde. In some cases, cells were pretreated with various agents (e.g., rabbit anti-CD44v₃ antibody, preimmune rabbit IgG, 20 μ g/ml cytochalasin D, 20 μ M W-7, or 1×10^{-5} M colchicine) at room temperature for 30 min, followed by fixing with 2% paraformaldehyde. Subsequently, cells were incubated with fluorescein-conjugated rat anti-CD44 antibody (50 μ g/ml) or fluorescein-conjugated rat IgG (50 μ g/ml; as a negative control). These fluorescein-labeled cells were then rendered permeable by ethanol treatment and stained with rhodamine-conjugated rabbit anti-MMP-9 IgG. To detect

nonspecific antibody binding, fluorescein-labeled cells were incubated with rhodamine-conjugated preimmune rabbit IgG. No labeling was observed in such control samples.

The fluorescein- and rhodamine-labeled samples were examined with a confocal laser scanning microscope (MultiProbe 2001 Inverted CLSM system, Molecular Dynamics, Sunnyvale, CA).

Binding of ¹²⁵I-labeled ankyrin/fodrin/spectrin to synthetic peptide and scrambled peptides

Nitrocellulose disks (1 cm in diameter) were coated with ≈ 1 μ g of either the ankyrin-binding region peptide (NGGNGTVEDRKPSEL) or a scrambled peptide (GRNETNPEGSGLDVK; synthesized by Dr. Eric Smith, University of Miami) at 4°C for 16 hr. After coating, the unoccupied sites on the disks were blocked by incubation with a solution containing 20 mM Tris-HCl, pH 7.4, and 0.3% bovine serum albumin at 4°C for 2 hr. The disks were then incubated with ¹²⁵I-labeled ankyrin/spectrin/fodrin ($\approx 3,000$ cpm/ng) at 4°C for 2 hr in 1 ml binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% bovine serum albumin). After binding, the disks were washed three times in the binding buffer and the disk-bound radioactivity was estimated. The nonspecific binding was determined in the presence of a 100-fold excess of one of the respective unlabeled ligands and subtracted from the total binding. Nonspecific binding was approximately 30% of the total binding. As a further control, the ligands were also incubated with uncoated nitrocellulose disks to determine the binding observed due to the "stickiness" of various ligands. Nonspecific binding was observed in these controls.

Binding of ¹²⁵I-labeled CD44v_{3,8-10} to ankyrin

Purified ¹²⁵I-labeled CD44v_{3,8-10} (≈ 0.32 nM protein, 1.5×10^4 cpm/ng) was prepared according to the procedures described previously (Bourguignon et al., 1993) and incubated with 30 μ l of ankyrin conjugated to sepharose beads (≈ 0.75 μ g protein) in 0.5 ml of the binding buffer (described above). Binding was carried out in the presence or absence of various concentrations (1 nM to 1 μ M) of unlabeled competing synthetic peptide (NGGNGTVEDRKPSEL, corresponding to the ankyrin binding sequence of CD44) (Lokeshwar et al., 1994) at 4°C for 5 hr under equilibrium conditions. Equilibrium conditions were determined by performing a time course (e.g., 1–10 hr) of the binding reaction. After binding, the beads were washed in the binding buffer and the bead-bound radioactivity was determined. Nonspecific binding was determined in the presence of either a 100-fold excess of unlabeled ankyrin or with bovine serum albumin-conjugated sepharose beads. Nonspecific binding was approximately 20–30% of the total binding and was subtracted from the total binding.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and Southern blot analysis

Total RNA was extracted from Met-1 cells by using the method described previously (Chomczynski and Sacci, 1987). Approximately 3 μ g of total RNA were used to synthesize first-strand oligo (dT)-primed cDNA at 42°C for 1 hr by an RT system (Promega, Madison,

WI) containing avian myeloblastosis virus RT. After first-strand synthesis, PCR amplification of cDNA was carried out with an initial melting of the RNA/cDNA hybrid at 94°C for 30 sec, annealing at 60°C for 30 sec, and polymerization at 72°C for 1 min. One pair of PCR primers was used to amplify between exon 5 and v3 (exon 7) to detect any splice variants of CD44 mRNAs containing v1, v2, or v3. Another pair was designed to amplify between v3 (exon 7) and exon 15 to detect CD44 mRNAs containing v3-10. The PCR primers used in this study were as follows: CD44 exon 5 left primer (5'-ACATCAGTCACAGACCTGCC-3'), CD44 exon 15 right primer (5'-ATCCATGAGTCACAGTGCGG-3'), CD44v3 (exon 7) right primer (5'-CTGAGGTGTCTGTCTTTC-3'), and CD44v3 (exon 7) left primer (5'-GACTCCACAGACAGAGAAAG-3'). A negative control was carried out in the absence of RT, and a positive control was carried out using human CD44v (v3, v8, v9, v10) cDNA as a template. The PCR products were examined on 2.0% agarose gel followed by Southern blot analysis hybridizing with human CD44 cDNA and/or mouse CD44 cDNA probes using the protocol previously described (Iida and Bourguignon, 1995). The PCR products were also one-step cloned using a TA-cloning kit (Invitrogen, San Diego, CA) and sequenced by the dideoxy sequencing method (Iida and Bourguignon, 1995).

Zymography and immunoprecipitation

Gelatin zymography was used with a modified procedure by Herron et al., 1986; Gunja-Smith et al., 1996) for detecting picograms of MMP-2 and MMP-9 and nanograms of other MMPs and proteases. SDS-PAGE was performed in 7.5% or 10% polyacrylamide containing 0.33 mg/ml gelatin. The gels were then rinsed twice in 0.25% Triton X-100 and incubated in the assay buffer [0.05 M Tris-HCl, pH 7.5, 0.2 M NaCl, 0.01 M CaCl₂, 1 μM ZnCl₂, 3 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% NaN₃, and 0.005% Brij 35] at 37°C for 18 hr. Gels were then stained with Coomassie blue R250. Both latent and active forms of gelatinases or other MMPs produce clear areas in the gel. Each gel lane contained preestimated amount of MMPs.

Immunoprecipitation analysis was performed according to standard methods. Serum-free media, Met-1 membrane fractions (Lokeshwar and Bourguignon, 1991, 1992; Lokeshwar et al., 1996), or anti-CD44-associated immune complexes were collected and analyzed for gelatinases or other MMP(s) content by zymography or substrate assays. In addition, conditioned serum-free media or Met-1 membrane fractions containing MMP(s) were immunoprecipitated with monospecific rabbit anti-MMP-9 IgG(s) using protein A-agarose suspensions (50–95 μg of protein A) at 4°C. A negative control, normal rabbit IgG or preimmune serum (in which endogenous MMPs, especially gelatinases, were removed by gelatin-sepharose affinity columns) was used. After the reacted agarose gels were washed three times with buffer (0.05M Tris-HCl, pH 7.5, 0.2 M NaCl, 0.01 M CaCl₂, 1 μM ZnCl₂, 3 mM PMSF, 0.02% NaN₃, and 0.005% Brij 35), the immune complexes were analyzed by zymography for specific enzyme activity or for proteins by SDS-PAGE.

The Met-1 membrane fraction or conditioned serum-free media were also used to quantitate for the MMP-

2 or MMP-9 gelatinase (MMPs) activity using ³H-acylated type I gelatin (Sellers et al., 1978; Gunja-Smith et al., 1996). Assays were set up in the presence or absence of 1.0 mmol/L aminophenylmercuric acetate (APMA) to measure latent and active enzymes; negative control samples containing 2 mmol/L 1,10-phenanthroline were also run in the assays.

Tumor cell migration assays

Twenty-four transwell units were used for monitoring in vitro cell migration, as described previously (Merzak et al., 1994). Specifically, the 8-μm porosity polycarbonate filters were used for the cell migration assay (Merzak et al., 1994). Met-1 cells [$\approx 1 \times 10^4$ cells/well in PBS, untreated or treated with various agents such as 20 μg/ml cytochalasin D, 20 μM W-7, 1×10^{-5} M colchicine, or 50 μg/ml rabbit anti-CD44v₃ antibody] were placed in the upper chamber of the transwell unit. The growth medium containing high glucose DMEM supplemented with 10% fetal bovine serum was placed in the lower chamber of the transwell unit. After 18 hr of incubation at 37°C in a humidified 95% air/5% CO₂ 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 migrated to the lower side of the polycarbonate filters by standard cell number counting methods, as described previously (Merzak et al., 1994). Each assay was performed in triplicate and repeated at least three times. All data were analyzed statistically by Student's t-test, and statistical significance was set at $P < 0.01$.

RESULTS

Expression of CD44 variant isoform(s) in Met-1 cells

The expression of CD44 variant isoforms is closely correlated with metastatic and proliferative behaviors of a variety of tumor cells including various carcinomas such as human breast tumor cells (Bourguignon et al., 1995; Iida and Bourguignon, 1995). To examine directly the CD44 isoform expression on the surface of Met-1 cells, we have used surface biotinylation techniques and a specific monoclonal anti-CD44 antibody that recognizes the CD44 epitope located at the N-terminus of the common domain (Iida and Bourguignon, 1995; Lokeshwar et al., 1996). Our results indicate that a single surface-biotinylated polypeptide (molecular weight ≈ 260 kDa) displaying immunological cross-reactivity with CD44 is preferentially expressed on the surface of Met-1 (Fig. 1A). This surface-labeled 260-kDa protein can also be immunoprecipitated (Fig. 1B) or immunoblotted (Fig. 1D) by rabbit anti-CD44v₃ antibody raised specifically against the v₃ sequence, suggesting that this protein is a CD44v₃-containing isoform. No CD44v₃-containing material is observed in control samples when either normal rat IgG or preimmune rabbit serum is used (Fig. 1F).

To verify the expression of CD44v₃-containing transcript(s) at the RNA level, total RNA from Met-1 cells was extracted and analyzed by RT-PCR using exon specific primers. Using a PCR primer pair to amplify between v3 (exon 7) and exon 15 by RT-PCR (Fig. 2C-a), a single product is detected at approximately 530 bp (Fig. 2A, lane 1). The size of the amplified fragment appears to correspond to a known CD44 variant isoform

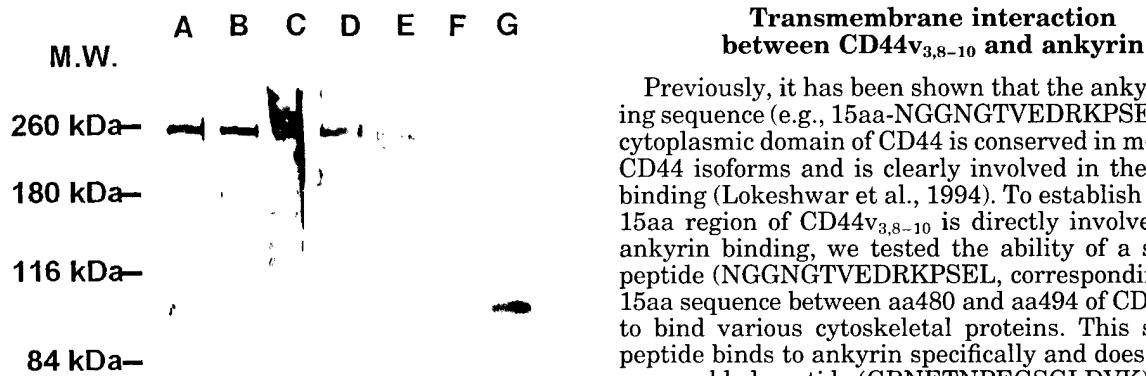


Fig. 1. Characterization of CD44 expression in Met-1 cells. **A:** Immunoprecipitation of surface biotinylated Met-1 cells using monoclonal anti-CD44 antibody (which recognizes the CD44 epitope located at the N-terminus of the common domain). **B:** Immunoprecipitation of surface biotinylated Met-1 cells with rabbit anti-CD44_{v₃}-specific antibody. **C:** Immunoprecipitation of Met-1 cells (metabolically labeled with $^{35}\text{SO}_4^{2-}$ with rabbit anti-CD44_{v₃}-specific antibody. **D:** Immunoblot of Met-1 cells with rabbit anti-CD44_{v₃}-specific antibody. **E:** Coimmunoprecipitation of MMP-9 with CD44: immunoprecipitation of Met-1 cells with rabbit anti-MMP-9 followed by immunoblot with rat anti-CD44 (which recognizes the CD44 epitope located at the N-terminus of the common domain). **F:** Immunoprecipitation of surface biotinylated Met-1 cells with normal preimmune rabbit serum (or normal rat IgG; data not shown). **G:** Immunoblot of Met-1 cells with rabbit anti-MMP-9 antibody.

that contains v3 and v8-10 exon insertions. Using a PCR primer pair to amplify between exon 5 and v3 (exon 7; Fig. 2C-b), one amplified DNA fragment is observed (about 270 bp, as shown in Fig. 2B, lane 1) that is identical to an amplicon from a human variant CD44 cDNA template containing v3, v8-10 exons (Fig. 2B, lane 2). The RT-PCR reaction is specific because no amplified fragment can be detected in samples without any RT (Fig. 2A, lane 2, 2B, lane 3). This v3-containing PCR product was subsequently "one-step cloned" into the pCR[®] vector from Invitrogen and sequenced. Our nucleotide sequence data indicate that only v3 (but not v1 or v2) is present in CD44 transcripts expressed in Met-1 cells and that the variant exon structure of CD44 transcripts containing v3 in Met-1 cells is the CD44_{v_{3,8-10}} isoform (Fig. 2C-c). This CD44_{v_{3,8-10}} variant exon structure was previously identified in human breast carcinoma samples (Bourguignon et al., 1995; Iida and Bourguignon, 1995) and its molecular mass (expressed at the protein level) has been shown to be ≈ 260 kDa (Bennett et al., 1995). Therefore, we believe that the major CD44_{v₃}-containing isoform expressed on the surface of Met-1 cells is the CD44_{v_{3,8-10}} isoform.

A number of CD44 isoforms have been shown to contain sulfated oligosaccharides (Bennett et al., 1995; Lokeshwar et al., 1996). Consequently, we metabolically labeled Met-1 cells with $^{35}\text{SO}_4^{2-}$ and then looked for the presence of radioactive sulfate label in CD44_{v_{3,8-10}} using anti-CD44_{v₃}-mediated immunoprecipitation (Fig. 1C). Our results indicate that $^{35}\text{SO}_4^{2-}$ is incorporated into CD44_{v_{3,8-10}} (possibly at the GAG chains; Fig. 1C). The GAG chains of certain CD44_{v₃}-containing isoforms may be involved in the binding of heparin binding growth factors, as shown previously (Bennett et al., 1995).

Previously, it has been shown that the ankyrin binding sequence (e.g., 15aa-NGGNGTVEDRKPSEL) in the cytoplasmic domain of CD44 is conserved in most of the CD44 isoforms and is clearly involved in the ankyrin binding (Lokeshwar et al., 1994). To establish that this 15aa region of CD44_{v_{3,8-10}} is directly involved in the ankyrin binding, we tested the ability of a synthetic peptide (NGGNGTVEDRKPSEL, corresponding to the 15aa sequence between aa480 and aa494 of CD44_{v_{3,8-10}}) to bind various cytoskeletal proteins. This synthetic peptide binds to ankyrin specifically and does not bind a scrambled peptide (GRNETNPEGSGLDVK) or other cytoskeletal proteins such as fodrin and spectrin (Table 1). Furthermore, we used competition binding assays and a synthetic peptide identical to the ankyrin binding domain (e.g., NGGNGTVEDRKPSEL) to analyze the ankyrin binding of CD44_{v_{3,8-10}}. Our results indicate that the synthetic peptide corresponding to the ankyrin binding domain of the CD44 isoform competes effectively with CD44_{v_{3,8-10}} in binding to ankyrin with an apparent inhibition constant (K_i) of approximately 0.7 nM (Fig. 3). These findings strongly suggest that an interaction between CD44_{v_{3,8-10}} and the cytoskeleton occurs at the cytoplasmic region of CD44_{v_{3,8-10}} (i.e., $^{480}\text{NGGNGTVEDRKPSE}^{494}\text{L}$) in Met-1 tumor cells.

Furthermore, we found that Met-1 cells are capable of forming membrane spikes or "invadopodia" structures (Fig. 5). Using in vitro migration assays, we found that CD44_{v_{3,8-10}}-containing Met-1 cells undergo active cell migration (Table 2). Treatments of Met-1 cells with various agents such as anti-CD44_{v₃} antibody, cytochalasin D (a microfilament-disrupting agent that prevents actin polymerization), and W-7 (a calmodulin antagonist), but not colchicine (a microtubule inhibitor) (Table 2) cause a significant inhibition of "invadopodia" formation and subsequent tumor cell migration (Table 2). These findings suggest that CD44_{v_{3,8-10}} and associated microfilament components play an important role in the regulation of both "invadopodia" formation and tumor cell motility.

Association of CD44_{v_{3,8-10}}, MMPs, and MMP-9 in Met-1 cells

Metastatic tumor cells are capable of degrading the ECM barrier to migrate out of the primary tumor location and into the circulation to establish a site of metastasis (Merzak et al., 1994; Boyd, 1996). The breakdown of the ECM can be traced to the action of one or more MMPs that belong to a family of zinc proteases (Woesner, 1991; Matrisian, 1992; Nagase, 1996). Normally, they are secreted as proenzymes and become activated outside the cell by serine proteases, such as plasmin, by the removal of a 9-kDa prosegment from the active site. Several MMPs, such as the 72-kDa gelatinase (gelatinase A, type IV collagenase, MMP-2), the 92-kDa gelatinase (gelatinase B, type V collagenase, MMP-9), the 57-kDa stromelysin (MMP-3), and interstitial collagenase (MMP-1), are likely to be responsible for ECM degradation during tumor invasion and metastasis (Stetler-Stevenson et al., 1993; Boyd, 1996).

In the present study, we employed a number of techniques, including zymographic assays, immunoblot/im-

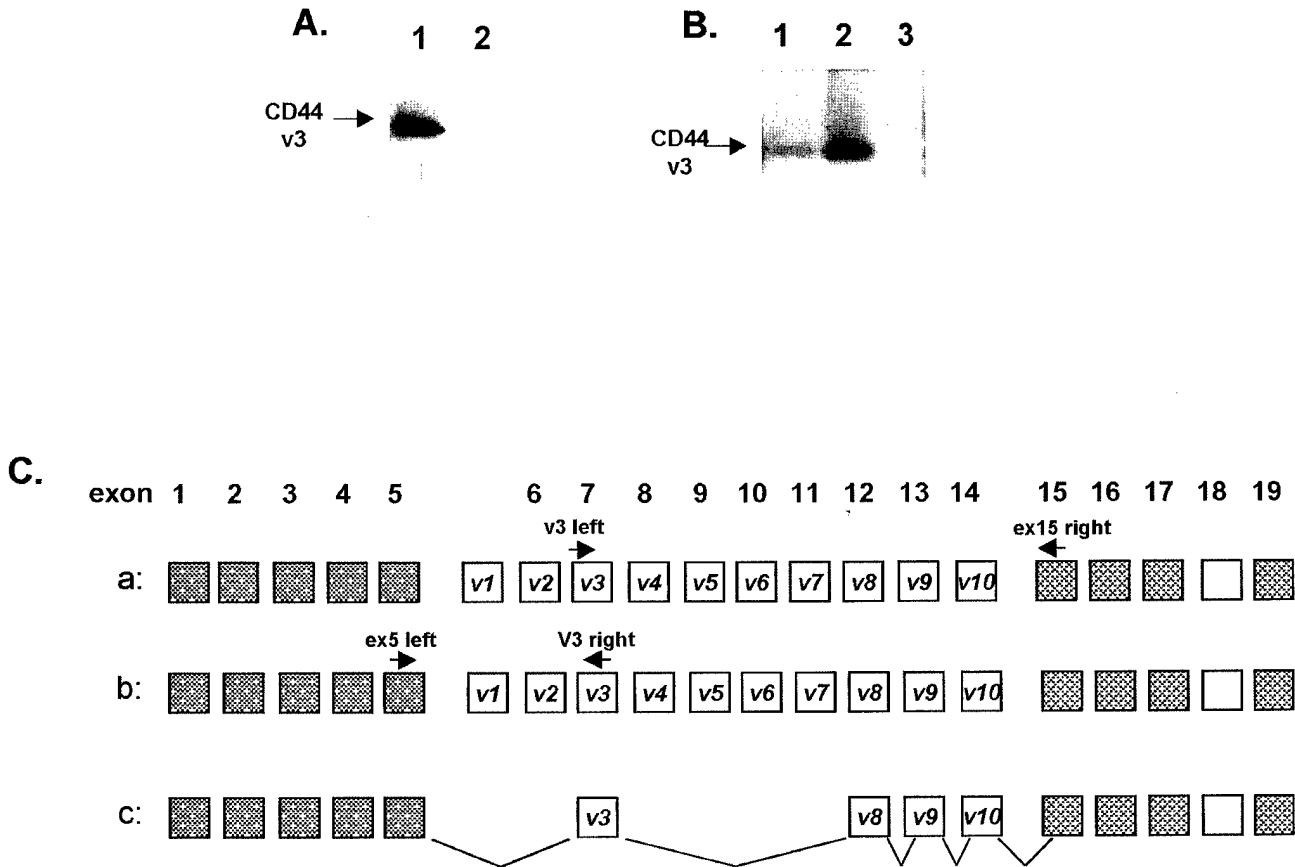


Fig. 2. RT-PCR and Southern blot analysis of CD44 transcript expression in Met-1 cells. Total RNA isolated from Met-1 cells were reverse-transcribed and subjected to PCR by using PCR primer pairs, as described in C-a and C-b. Subsequently, RT-PCR products were analyzed by Southern blot hybridization, cloning, and nucleotide sequence analysis as described in Materials and Methods. **A:** Southern blot analysis of a single RT-PCR product (≈ 530 bp) generated by CD44_{v3} (exon 7) and exon 15 primer pairs (the primer design is shown in C-a, lane 1). As a negative control, RT-PCR was carried out in the absence of RT (lane 2). **B:** Southern blot analysis of a single RT-PCR

product (≈ 270 bp) generated by CD44 exon 5 and v3 (exon 7) primer pairs (the primer design is shown in C-b, lane 1). As a positive control, a known CD44 variant isoform that contains v3 and v8–10 exon insertions was used (lane 2). As a negative control, RT-PCR was carried out in the absence of RT (lane 3). **C:** Schematic exon map of CD44 in mouse. **C-a:** Specific CD44_{v3} (exon 7) and exon 15 primer pairs used in A. **C-b:** Specific CD44 exon 5 and v3 (exon 7) primer pairs used in B. **C-c:** Schematic illustration of the CD44_{v3,8-10} isoform identified in Met-1 cells.

TABLE 1. Binding ($\text{nM} \times \text{cpm bound}$) of ^{125}I -labeled cytoskeletal proteins (ankyrin, spectrin, and fodrin) to synthetic peptides¹

Binding to NGGNGTVEDRKPSEL	
¹²⁵ I-labeled ankyrin	55,500 \pm 105
¹²⁵ I-labeled spectrin	1,020 \pm 32
¹²⁵ I-labeled fodrin	1,100 \pm 42
Binding to GRNETNPEGSGLDVK	
¹²⁵ I-labeled ankyrin	1,150 \pm 28
¹²⁵ I-labeled spectrin	1,090 \pm 21
¹²⁵ I-labeled fodrin	1,178 \pm 30

¹The ^{125}I -labeled cytoskeletal proteins (100 ng each of ankyrin, spectrin, and fodrin) were incubated with nitrocellulose disks coated with either the synthetic peptide NGGNGTVEDRKPSEL (corresponding to the sequence between aa480 and 494 of human CD44s) or the scrambled peptide GRNETNPEGSGLDVK at 4°C for 4 hr as described in Materials and Methods. Nonspecific binding was determined in the presence of a 100-fold excess of the respective unlabeled cytoskeletal proteins and subtracted from the total binding.

munoprecipitation techniques, and immunofluorescence staining to examine the relationship between gelatinase activities and the CD44_{v3,8-10} in Met-1 tumor cells. The results of the gelatin zymographic analyses

indicate that the lytic band of mouse gelatinase in the membrane fraction of Met-1 cells is approximately 92 kDa (Fig. 4A-a). No other lytic bands of gelatinase were observed. Using anti-human MMP-9-mediated immunoblot (Fig. 1G) and immunoprecipitation (Fig. 4A-b) of Triton X-100 solubilized Met-1 membranes, we found that this 92-kDa protein displays lytic gelatinase activities corresponding to the lytic band of human MMP-9 (Fig. 4B-d). As a control, the zymographic profile of anti-MMP-9 IgG alone (without using Triton X-100 solubilized Met-1 membranes and immunoprecipitation) shows no lytic bands of gelatinase (Fig. 4A-d). This result suggests that the lytic band of 92-kDa gelatinase detected in the membrane fraction of Met-1 cells (Fig. 4A-b) corresponds to a MMP-9-like molecule.

It has been determined that the lytic band corresponding to the latent form of normal mouse MMP-9 is approximately 105 kDa (Reponen et al., 1994; Tanaka et al., 1993; Fig. 4B-a). Our data indicate that the 92-kDa gelatinase from Met-1 membrane fraction

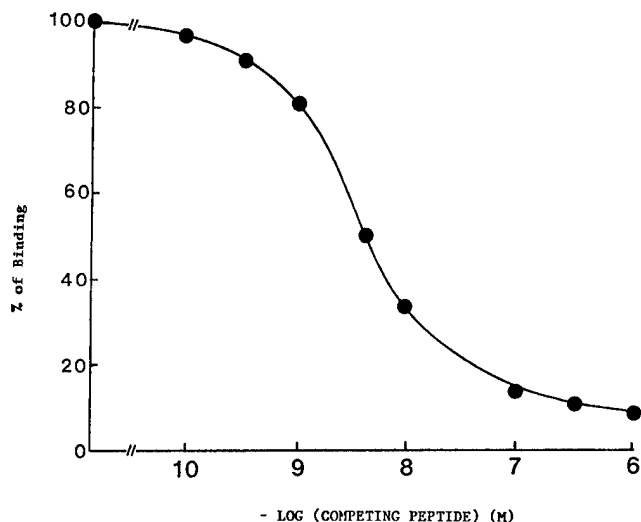


Fig. 3. Binding of ^{125}I -labeled CD44 $v_{3,8-10}$ to ankyrin. ^{125}I -labeled CD44 $v_{3,8-10}$ was incubated with ankyrin in the presence of different concentrations of unlabeled synthetic peptide (NGGNGTVEDRKPS-SEL) corresponding to the ankyrin binding domain of CD44 as described in Materials and Methods. The specific binding observed in the absence of any of the competing peptides is designated as 100%. The results represent an average of duplicate determinations for each concentration of the competing peptide used.

TABLE 2. Effects of different drugs on "invadopodia" formation and cell migration

Treatments ¹	"Invadopodia" (% of control) ²	Cell migration ³
No treatment (control)	100	100
Preimmune serum	96	98
Anti-CD44 v_3 antibody	15	23
Cytochalasin D	28	22
W-7	18	16
Colchicine	98	97

¹The concentrations of reagents used in this experiment were 50 $\mu\text{g}/\text{ml}$ rabbit anti-CD44 v_3 , 20 $\mu\text{g}/\text{ml}$ cytochalasin D, 20 μM W-7, and 1×10^{-5} M colchicine.

²The values expressed represent an average of triplicate determinations of 3–5 experiments with a standard deviation of less than $\pm 5\%$.

³In these experiments, ≈ 30 – 40% of input cells ($\approx 1 \times 10^4$ cells/well) underwent in vitro migration in the control samples.

appears to be slightly smaller than the normal latent form of mouse MMP-9 (105 kDa; Fig. 4B-b). The reduced molecular weight of MMP-9 of Met-1 suggests that this gelatinase is in an active form. Treatment of the Met-1 membrane fraction with $\alpha(2)$ -macroglobulin (an inhibitor that binds active metalloprotease activity) (Nagase et al., 1994) results in blockage of the lytic reaction of 92-kDa gelatinase activity (Fig. 4B-c). Measurement of the gelatinase activity using ^3H -acetylated type I gelatin (Sellers et al., 1978; Gunja-Smith et al., 1996) indicates that the MMP-9 associated with Met-1 membranes is an active gelatinase. This enzyme does not require APMA to become active, and its activity can be readily inhibited by 1,10-phenanthroline (data not shown). These findings further support the notion that the lytic band of the 92-kDa mouse MMP-9 associated with Met-1 membranes is present in a proteolytically active form. In addition, we observed a broad lytic band (possibly a secreted form of MMP-9)

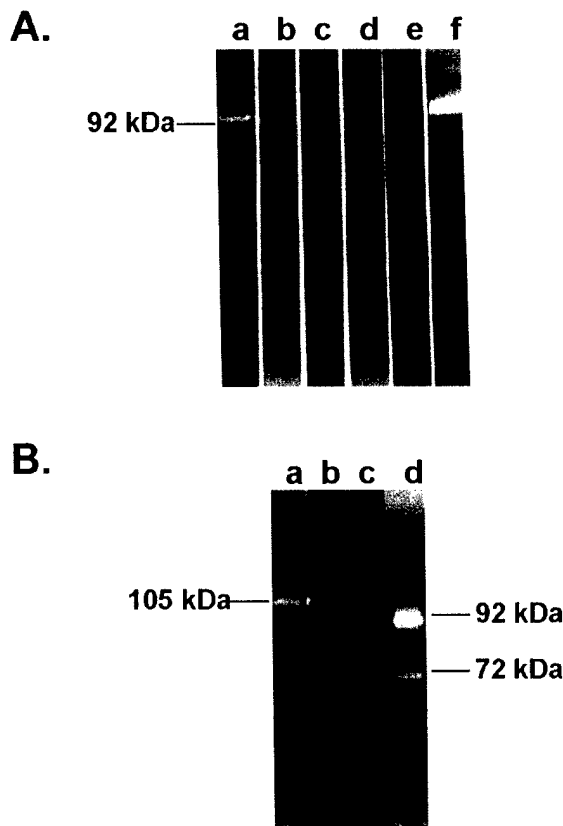


Fig. 4. Gelatin zymographic analysis of MMPs in Met-1 cells. Gelatin zymography was used to detect MMPs in Met-1 cells according to the procedures described in Materials and Methods. **A-a:** The 92-kDa lytic gelatinase band detected in Met-1 membranes. **A-b:** The human MMP-9 (92-kDa lytic gelatinase band) detected by anti-human MMP-9-mediated immunoprecipitation of Triton X-100 solubilized Met-1 membranes. **A-c:** The 92-kDa lytic gelatinase band associated with the anti-CD44-immunoprecipitated materials. **A-d:** As a control, anti-MMP-9 IgG was used (in the absence of Triton X-100 solubilized Met-1 membranes and immunoprecipitation). **A-e:** As a control, anti-CD44 IgG was used (in the absence of Triton X-100 solubilized Met-1 membranes and immunoprecipitation). **A-f:** The secreted form of MMP-9 (containing both latent, 105 kDa, and active, 92 kDa, mouse gelatinase) collected from the serum free conditioned media containing Met-1 cells. **B-a:** The lytic band (≈ 105 kDa) corresponding to the latent form of normal mouse MMP-9. **B-b:** The 92-kDa gelatinase from Met-1 membrane fraction. **B-c:** Treatment of the Met-1 membrane fraction with $\alpha(2)$ -macroglobulin (an inhibitor known to inactivate metalloprotease activity). **B-d:** Human MMP-9 (92 kDa) and MMP-2 (72 kDa) markers.

in the serum-free conditioned media from Met-1 cells, which may represent the lytic bands of both the latent (105 kDa) and active (92 kDa) mouse gelatinase (Fig. 4A-f).

Further analyses by double immunofluorescence labeling and confocal microscopy indicate that the 92-kDa mouse MMP-9-like molecule (Fig. 5B) is colocalized with CD44 $v_{3,8-10}$ (Fig. 5A) in the "invadopodia" structure of the Met-1 cells. No CD44 $v_{3,8-10}$ or MMP-9 staining was observed in control samples when either normal rat IgG (Fig. 5a) or preimmune rabbit IgG (Fig. 5b) was used. To explore the possible association between these two molecules, we carried out anti-human MMP-9-mediated immunoprecipitation of Triton X-100 solubilized Met-1 membranes followed by immunoblot-

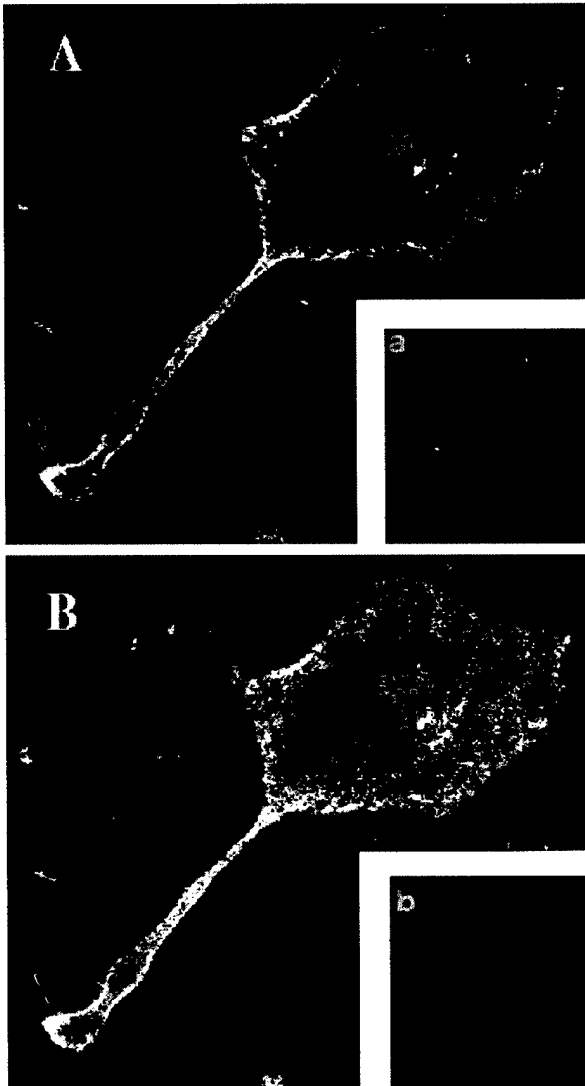


Fig. 5. Double immunofluorescence staining and confocal analysis of "invadopodia" structures (arrowheads) in Met-1 cells. **A:** Immunostaining of surface CD44_{v_{3,8-10}} using fluorescence-labeled rat anti-CD44 IgG. **a:** A negative control of surface CD44_{v_{3,8-10}} staining using fluorescence-labeled normal rat IgG. **B:** Immunostaining of intracellular MMP-9 using rhodamine-labeled rabbit anti-MMP-9 IgG. **b:** A negative control of intracellular MMP-9 staining using rhodamine-labeled preimmune rabbit IgG.

ing with anti-CD44_{v₃} antibody. Our data show that CD44_{v_{3,8-10}} is coprecipitated with the metalloprotease (e.g., MMP-9) as a complex (Fig. 1E). In addition, we carried out anti-CD44-mediated precipitation followed by gelatin zymography analysis. Our results indicate that the 92-kDa lytic gelatinase band can be detected in the anti-CD44-immunoprecipitated materials (Fig. 4A-c). As a control, the zymographic profile of anti-CD44 IgG (without using Triton X-100 solubilized Met-1 membranes and immunoprecipitation) shows no lytic bands of gelatinase (Fig. 4A-e). These findings suggest that CD44_{v_{3,8-10}} and 92 kDa are tightly associated with each other.

DISCUSSION

Several studies have indicated that the expression of CD44 on the cell surface changes profoundly during tumor metastasis, particularly during the progression of various carcinomas (Arch et al., 1992; Bourguignon et al., 1995; Iida and Bourguignon, 1995). Many tumor cells and tissues have been found to express different CD44v isoforms (Arch et al., 1992; Bourguignon et al., 1995; Droll et al., 1995; Iida and Bourguignon, 1995). These isoforms, containing additional exon insertions (e.g., exons 6–11) within the CD44 membrane proximal region, appear to be associated with tumor formation (Iida and Bourguignon, 1997) and metastasis (Arch et al., 1992; Bourguignon et al., 1995; Iida and Bourguignon, 1995). In fact, certain CD44v expression has been used as an indicator of metastasis. In the present study, we chose Met-1 cells, which were derived from high metastatic potential tumors, as a model system to analyze the relationship between CD44 isoform expression and breast tumor progression. Immunological analyses indicated that the CD44 isoform (≈ 260 kDa) expressed in Met-1 displays strong cross-reactivity with several anti-CD44 antibodies including rat anti-human CD44 antibody (clone 020; Fig. 1A), rabbit anti-CD44v3 antibody (Fig. 1B), and two other anti-mouse CD44 antibodies (e.g., IM-7 and IRWB-14; data not shown). Further analyses using RT-PCR, Southern blot, and nucleotide sequence techniques indicated that this 260-kDa CD44 isoform contains a v_{3,8-10} exon insertion. CD44v₃-containing isoforms, including CD44v_{3,8-10}, have been shown to be preferentially expressed on highly malignant breast carcinoma tissues but not on normal or noninvasive breast cancer tissues. In fact, there is a direct correlation between CD44v_{3,8-10} isoform expression and increased histologic grade of the malignancy (Iida and Bourguignon, 1995; Sinn et al., 1995; Kalish et al., 1997). These findings suggest 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; Iida and Bourguignon, 1995; Kalish et al., 1997; Kaufmann et al., 1995). Therefore, it is not surprising that CD44v_{3,8-10} was the predominant CD44 species detected on the surface of these highly metastatic Met-1 cells. Because information regarding the role of CD44v_{3,8-10} in regulating mammary tumor cell function is very limited at the present time, it is clearly important to examine the structural properties of this molecule.

CD44v_{3,8-10} (with heparan sulfate, or GAG, addition) has been shown to bind bFGF on COS cells (Bennett et al., 1995). Preliminary data indicate that the external domain of sulfated CD44v_{3,8-10} (Fig. 2C) in Met-1 cells preferentially binds vascular epithelial growth factor (VEGF), but not bFGF (data not shown). VEGF is a specific mitogen for endothelial cells and a potent microvascular permeability factor (Folkman, 1985; Dvorak et al., 1995). It plays an integral role in angiogenesis and thus in potentiation of solid tumor growth (Folkman, 1985; Dvorak et al., 1995). Recent data indicate that Met-1 cells are capable of inducing a high level of intratumoral microvessel formation (Young et al., 1995; Cheung et al., 1997). Therefore, the attachment of VEGF to the heparin sulfate sites on CD44v_{3,8-10} may

be responsible for the onset of breast tumor-associated angiogenesis.

A number of different MMPs, including the 72-kDa gelatinase (gelatinase A, type IV collagenase, MMP-2), the 92-kDa gelatinase (gelatinase B, type V collagenase, MMP-9), the stromelysins (MMP-3, MMP-11), and the interstitial fibroblast-type collagenase (MMP-1), are thought to play an important role in degrading ECM materials during tumor invasion and metastases (Merzak et al., 1994; Boyd, 1996; Chen, 1996). Biochemical interactions between MMPs and various cell surface molecules have not been fully established. A membrane-type MMP, or transmembrane MMP, and TIMP-2, a tissue inhibitor of MMP-2 have been reported to be involved in the activation of MMP-2 on the cell surface (Polette et al., 1996). Brooks et al. (1996) determined that MMP-2 is localized in a proteolytically active form on the surface of invasive cells based on its ability to bind directly to integrin $\alpha v \beta 3$. The question of whether other MMPs are also involved in interacting with CD44-related adhesion molecules during the progression and metastasis of tumors remains to be answered.

In the present study, we used anti-CD44 or anti-MMP-9-mediated immunoblot/immunoprecipitation and gelatin zymography to demonstrate the possible physical association between these two molecules. Specifically, we found that CD44_{v3,8-10} is closely associated with MMP-9 (gelatinase B) in the plasma membrane of Met-1 cells based on the evidence provided by anti-MMP-9 and anti-CD44-mediated immunoprecipitation followed by immunoblot and gelatin zymography (Figs. 1E, 4A-c). Furthermore, CD44_{v3,8-10}-associated MMP-9 appears to be present in a proteolytically active form (Fig. 4B-b) and preferentially localized at the "invadopodia" structure of the Met-1 cells (Fig. 5). Our results are consistent with previous findings showing that certain proteases are localized on "invadopodia" of human malignant melanoma cells (Chen, 1996). Therefore, we believe that the close interaction between CD44_{v3,8-10} and the active form of MMP-9 in the "invadopodia" structure of Met-1 tumor cells may be required for the degradation of ECM for tumor cell invasion and metastasis. Preliminary data indicate that MMP-9 is complexed with the cytoplasmic domain (not the extracellular portion) of the CD44_{v3,8-10} molecule (data not shown). The nature of the linkage between these two molecules is currently undergoing investigation.

The invasive phenotype of tumor cells characterized by "invadopodia" formation (Mueller and Chen, 1991; Monsky et al., 1994) and tumor cell motility (Jiang et al., 1994; Lauffenburger and Horwitz, 1996) has been linked to cytoskeletal function. Dissection of the transmembrane pathways controlling these cellular processes should aid in understanding the regulatory mechanisms underlying tumor invasion and metastasis. Previously, we have demonstrated that CD44 isoforms (e.g., CD44s and CD44v ex14/v10) display certain high affinity binding to the cytoskeletal protein ankyrin (Bourguignon et al., 1991, 1993, 1995; Lokeshwar and Bourguignon, 1991, 1992; Lokeshwar et al., 1994, 1996; Bourguignon, 1996). The cytoplasmic domain of the CD44 isoforms contain the ankyrin binding site (NGNGTVEDRKPSEL), which is $\geq 90\%$ conserved in all of the CD44 isoforms (Lokeshwar et al., 1994) and resides between amino acids 480 and 494 of the

CD44_{v3,8-10} molecule. This ankyrin binding sequence appears to be required for regulating CD44 isoform-mediated function (Bourguignon et al., 1995; Bourguignon, 1996). The fact that the synthetic peptide (NGNGTVEDRKPSEL), which corresponds to the ankyrin binding domain of the CD44 isoform, competes effectively with CD44_{v3,8-10} to bind ankyrin ($K_i \approx 0.7$ nM; Fig. 3) suggests that the sequence of NGNGTVEDRKPSEL in the cytoplasmic domain of CD44_{v3,8-10} interacts directly with ankyrin. Most importantly, we have found that treatments of Met-1 cells with certain agents including anti-CD44_{v3,8-10}, cytochalasin D (a microfilament inhibitor), and W-7 (a calmodulin antagonist), but not colchicine (a microtubule-disrupting agent) effectively inhibit "invadopodia" formation and tumor cell migration (Table 2). Therefore, we believe that CD44_{v3,8-10} plays an important role in linking ankyrin to the membrane-associated actomyosin contractile system required for "invadopodia" formation, tumor cell migration, and matrix degradation during breast cancer progression.

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(9) APPENDICES

9. 2. Manuscript

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EXPRESSION OF MATRIX METALLOPROTEINASE-9 (GELATINASE B) AND A TISSUE INHIBITOR OF METALLOPROTEINASE (TIMP-1) IN HUMAN BREAST ADENOCARCINOMA

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The deadly consequences of breast cancer are largely due to metastasis, a process in which tumor cells penetrate blood vessels and enter other tissues. This movement through vessels and tissues is attributed to matrix metalloproteinases [MMPs] that destroy the matrix. The small amounts of MMPs produced in normal tissues are held in check by tissue inhibitors, but increased production of MMPs or decreased production of the tissue inhibitors [TIMPs] may be implicated in tumor invasion and metastasis (1,2). In this study we used zymography, immunohistochemistry and mRNA assays to assess the levels of MMP-9 and TIMP-1 in the same sample of breast tissue. The different tissues analyzed included tissues that were biopsied as normal, or as benign [fibroadenoma] tumors, or as noninvasive [*in situ* ductal and *in situ* lobular] or as invasive [infiltrating or poorly differentiated ductal, lobular, medullary, and colloidial] carcinomas.

Analysis of extracts [100 -150 mg] of breast tissue by gelatin and transferrin zymography (3) revealed the presence of latent [92 kDa] and active [82 kDa and 68 kDa] MMP-9, latent [72 kDa] and active [62 kDa] MMP-2 and higher molecular weight aggregates of MMP-2 and -9. Quantification of MMP bands showed a general increase in MMPs in all cancer tissues. However, the increase in MMP-9 was the most striking, as it was present at levels 20 times [*in situ* ductal] to 200 times [infiltrating ductal] in carcinoma tissues compared to the low levels [often undetectable] found in normal tissues and benign tumors. Zymography also showed that a greater proportion of the latent MMP-9 and MMP-2 was present in their active forms in carcinoma tissues.

Reverse zymography (3) demonstrated the presence of TIMPs -1,-2 and -3 in all breast tissues, but quantitation of TIMP-1 [ELISA kit] clearly showed that the level of TIMP-1 was much lower in invasive cancer tissues compared to normal and benign tissues.

Immunohistochemical (4) staining of adjacent paraffin-embedded tissue sections (3 μ) with anti-MMP-9 or anti-TIMP-1 IgGs indicated that MMP-9 and TIMP-1 were produced by tumor cells and by stromal component of the breast tissue. Normal breast tissues showed the expression of

TIMP-1 [3 - 4⁺ staining] in the stroma and occasional low staining for MMP-9 in epithelial cells. Benign neoplasm sections exhibited 2⁺ staining for TIMP-1 in the stroma and occasional staining [epithelial cells] for MMP-9. Carcinoma tissues of all grades and types showed weak to 1⁺ staining for TIMP-1 in stroma and strong staining for MMP-9 [3 - 4⁺] in tumor cells. MMP-9 was found to be expressed [cytoplasmic staining] mainly in tumor cells.

Reverse transcription polymerase chain reaction [RT-PCR] analysis (5,6) was used as an assay for semi quantitation of low-abundance mRNAs of MMP-9 and TIMP-1 gene expression in breast tissues. Total RNA was isolated [GIBCO BRL kit] and 5' and 3' sequence-specific oligonucleotide primers were used to amplify 640 bp for MMP-9 cDNA, 551 bp for TIMP-1 cDNA and 230 bp for house keeping gene-GAPDH cDNA. The RT-PCR analysis showed a higher expression of MMP-9 in cancer tissues, the highest expression being noted in infiltrating and undifferentiated ductal carcinomas. In contrast, TIMP-1 expression was found to be lower in these carcinomas than in benign tissues .

This unified multipronged [biochemical, immunohistochemical and molecular biological techniques] study confirms that there is an elevated expression of MMP-9 and an underexpression of TIMP-1 in breast carcinoma tissues. In normal tissue, TIMP-1 has a dual role of complexing with latent MMP-9 to avoid its activation (7) and also to bind with the active enzyme and inhibit the enzyme action, thus limiting the degradation of the surrounding matrix. The observed imbalance of MMP-9 over tissue inhibitor (TIMP-1) in carcinoma tissue would lead to an increased matrix degradation and contribute to the invasiveness or metastatic potential of human breast carcinomas.

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(9) APPENDICES

9. 3. Manuscript

ROLE OF MATRIX METALLOPROTEINASES AND THEIR TISSUE INHIBITORS IN HUMAN ADENOCARCINOMA.

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Excessive expression of a group of matrix metalloproteinases (MMPs) or decreased production of their tissue inhibitors (TIMPs) have been implicated in tumor invasion and metastasis. In this study we have undertaken a unified multipronged approach by examining enzyme (MMPs), inhibitors (TIMPs) levels by zymography, together with immunohistochemical identification of the types involved and mRNA levels to show the expression or repression of MMPs and TIMPs in the biopsied breast tissues. The breast tissues (eighty samples) consisting of normal, benign tumors (fibroadenoma, phyllodes tumor; fibrocystic changes) and noninvasive (insitu ductal and insitu lobular) or invasive or infiltrating (ductal, lobular, medullary, colloidal, tubular) carcinomas were analyzed. The cancer tissues were further identified for nuclear grades II or III from pathological reports. Specific MMPs (MMP-1 or 13, interstitial collagenase; MMP-2, (gelatinase A; MMP-9 (gelatinase B); MMP-7 (matrilysin); MT-MMP (membrane type I MMP) and stromelysin-1 together with specific TIMPs (TIMP-1, -2 and -3) were analyzed. Use of SDS-PAGE-gelatin or transferrin zymography identified and quantitated (with an Imager software program) MMPs. MMP-1 and gelatinase A and B were also quantitated by tritiated-substrate assays. Reverse gelatin zymography and TIMP-1 ELISA kit was used to locate and quantitate the TIMPs. Using specific anti-MMPs and anti-TIMPs IgGs, and hematoxylin and eosin stain, immunohistochemical evaluations (staining score of 1-4) of sections (3 microns) of adjacent paraffin embedded tissue blocks were carried out to locate and identify the expression of MMPs and TIMPs in breast pathology.

Keywords: Metalloproteinases (MMPs); Tissue Inhibitors of MMPs (TIMPs); Gelatinases; Immunohistochemistry; Breast Tumors.

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Extracts (0.25% Triton-X 100, 1 M GuHCL and 0.1% SDS in standard Tris buffer) of small amounts (100 -500 mg) of breast tissue analyzed by gelatin and transferrin zymography showed the presence of latent and active MMP-2 (72 kDa, 62 kDa) and MMP-9 (92 kDa, 82 kDa, 68 kDa), higher molecular weight aggregates of MMP-2 and -9, serine proteinases and in some tissues MMP-1 (55 kDa). Biochemical quantification of MMPs showed an overall increase in all types of MMPs in cancer tissues. MMP-9 was the key MMP; it was present at levels 0.23 (*in situ* ductal) to 2.3 (infiltrating ductal) $\mu\text{g/g}$ wet weight tissue in cancer tissues compared to unquantifiable amounts in normal (3/6) and benign neoplasm (11/16) tissues. There was a correlation of constitutively expressed MMP-1 (0.04 - 0.66 $\mu\text{g/g}$ tissue) and MMP-2 (0.23 -1.7 $\mu\text{g/g}$ tissue) in cancer tissues. Zymography also showed a fraction of MMP-9 and MMP-2 in their active forms in grade III breast cancers compared to normal and benign tissues.

Reverse zymography showed the presence of TIMPs -1,-2 and -3 in all breast tissues. Quantitation by TIMP-1 ELISA kit and reverse zymography clearly showed that total amounts of TIMPs were lower in cancer (0.2 and 1.0 $\mu\text{g/g}$ wet weight tissue in grade III and II, respectively) compared to higher amounts in normal (4.5 μg) and benign (3.5 μg) and noninvasive cancer (*in situ* ductal carcinoma, 1.5 μg) tissues. The production of TIMPs in cancer tissues falls far short of the amount needed to counteract the excessive production of MMPs leading to an imbalance in enzyme and inhibitor expression.

Immunohistochemical evaluations of the adjacent paraffin-embedded tissue sections (3 μ) showed the direct production of MMP-9, MMP-2, MMP-1, MMP-7 and TIMP-1, -2 and -3 by various cell types; tumor cells or neighboring stromal cells. Sections of normal breast tissues showed the expression of MMPs and TIMPs mainly in the stromal component of the tissues with occasional staining of ductal epithelial cells. Only weak staining with anti-MMP-9 IgGs was seen in stroma. Normal tissues stained highly (3-4⁺ staining) for TIMPs (stroma only) and stained 1⁺ for MMP-2 and MMP-1. Benign neoplasm sections exhibited the equal staining (2-3⁺) of epithelial cells and stroma with MMP-2, MMP-1 and MMP-7 (weak stain) IgGs; stroma only with TIMP-1 and TIMP-2 IgGs (1⁺); stroma and tumor cells with TIMP-3 IgGs and occasional staining (tumor cells) with MMP-9 IgGs. Cancer tissues of all grades and types showed staining with MMP-9 (3-4⁺), MMP-2, MMP-1 and MT1-MMP IgGs. Weak to 1⁺ staining with TIMP-1, TIMP-2 and TIMP-3 IgGs was seen in stroma and in tumor cells. MMP-9 was found to be expressed (cytoplasmic staining) mainly by tumor (epithelial) cells.

This multipronged (biochemical and immunohistochemical) study categorically shows that there is an elevated expression of MMP-9 together with overexpression of constitutively expressed MMP-2 and MMP-1; and underexpression of TIMPs -1, -2 -3 by both stroma and tumor cells in all types of breast cancer. This imbalance of MMPs over their tissue inhibitors (TIMPs) may contribute to the invasiveness or metastatic potential of human breast cancer.

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